

ORIGINAL ARTICLE

RNAi-mediated COPS3 gene silencing inhibits metastasis of osteogenic sarcoma cells

T Yan¹, G Tang¹, T Ren, D Shen, K Sun, W Liang and W Guo

Musculoskeletal Tumor Center, Peking University People's Hospital, Beijing China

Metastatic disease is the primary cause of mortality among patients with osteogenic sarcoma (OGS). In this study, we aimed to identify the relationship of COPS3 gene expression to metastasis. Immunohistochemical staining for COPS3 was performed on 65 OGS samples (37 without and 28 with metastatic disease); 18.9% (7/37) of specimens from patients with no metastasis and 57.1% (16/28) of specimens from patients with metastasis showed intense staining of COPS3. Comparison of COPS3 expression between a poorly metastatic osteosarcoma cell line (SAOS-2) and highly metastatic osteosarcoma cell line (HOS) showed stronger expression of COPS3 in HOS cells. Inhibiting COPS3 function by siRNA resulted in reduced proliferation and migration of HOS cells. Inhibition of COPS3 gene downregulated expression of the MAPK signaling pathway, which has an important role in metastasis of OGS. Our results suggested that overexpression of the COPS3 gene might have important roles in metastasis of osteosarcoma cells. *Cancer Gene Therapy* (2011) 18, 450–456; doi:10.1038/cgt.2011.16; published online 25 March 2011

Keywords: COPS3; osteogenic sarcoma; metastasis; immunostaining; siRNA

Introduction

Osteogenic sarcoma (OGS) is the most common primary malignant tumor of the bone. It is characterized by a local aggressiveness, with a tendency to metastasize to the lung and bone in children and young adolescents.^{1,2} Approximately 10–20% of patients have metastases at the time of diagnosis.³ Without adjuvant chemotherapy, more than half of the patients treated by surgery alone develop metastases within 6 months and more than 80% develop recurrent disease within 2 years of diagnosis.⁴ The use of multiagent chemotherapy, in addition to surgery, has dramatically increased long-term survival for patients with OGS, with a disease-free 5-year survival reaching 65–70% for patients with localized disease.^{5,6} However, patients who develop metastatic disease still have an extremely dismal prognosis. A 5-year survival for patients with metastatic OGS remains as low as 20%, even with the introduction of chemotherapy three decades ago. Metastasis remains the primary cause of poor survival of patients with OGS.

Metastasis development is a complex and multistage process involving local invasion, access to the circulation, seeding and eventual proliferation within a favorable

distant target organ.^{7,8} Several genes, including *WT1*, *CXCR4*, *TEM7* and *PTHRI*,^{9–12} have been recently reported to be associated with metastasis of OGS. Khanna *et al.*¹³ reported that the membrane-cytoskeleton linkage protein Ezrin is necessary for OGS metastasis and that there is a significant association between high Ezrin expression and poor outcome in pediatric OGS patients. Despite these recent advances, the process of metastasis still remains incompletely characterized at both the molecular and biochemical levels.

Amplification of several genes that map to a region of chromosome 17p11.2, including COPS3, was observed in high-grade osteosarcoma. Comparative genomic hybridization studies revealed amplification of this region in 13–29% of high-grade osteosarcomas,^{14,15} suggesting the presence of an oncogene (or oncogenes) whose activation may contribute to osteosarcoma tumorigenesis. In studies with small sample sizes, COPS3 had been found to be amplified in 32–63% of osteosarcoma specimens and was also shown to be overexpressed and potentially involved in osteosarcoma tumorigenesis.^{16,17} Recently, it has been shown that COPS3 amplification strongly correlates with large tumor size ($P=0.0009$).¹⁸ In the clinical setting, tumor volume is reportedly associated with lung metastasis in patients with osteosarcoma.¹⁹ We hypothesize that COPS3 could regulate OGS growth and metastasis and might correlate with poor patient outcome.

In this report, we evaluated immunohistochemically the expression pattern of COPS3 in OGS specimens and correlated these findings with the occurrence of metastasis. We then compared COPS3 expression between OGS cell lines with different metastasis potentials and

Correspondence: Professor W Guo, Musculoskeletal Tumor Center, Peking University People's Hospital, No.11 Xizhimen South Street, Beijing 100044, China.

E-mail: bonetumor@163.com

¹These authors contributed equally to this work.

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developed an siRNA-based approach to inhibit COPS3 expression. Finally, we evaluated the roles of COPS3 in migration of the osteosarcoma cell line.

Materials and methods

Patient specimens

A study of 65 patients with OGS, undergoing surgery at the Musculo-Skeletal Tumor Center of Peking University People's Hospital, Beijing, China, during the period 1997–2008, was performed. The median age at diagnosis was 19 years (range 5–79). Primary histological examination was performed at the Department of Pathology, Peking University People's Hospital. Histologic tumor slides were reevaluated by two pathologists. All patients were free of metastatic disease at the time of diagnosis. Treatment protocols of these patients were in the same fashion. In all cases, Institutional Review Board-approved protocols were followed to collect specimens, and the study had the approval from the Hospital Research Ethics Committee.

Cell culture and reagents

OGS-derived cell lines (HOS and SAOS-2) were obtained from American Type Cell Collection (Manassas, VA) and cultured in 1640 medium containing 10% fetal bovine serum, 1% antibiotics–antimycotics (Invitrogen, Carlsbad, CA) in a 5% CO₂-humidified atmosphere at 37°C. Anti-COPS3 and anti-actin were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-MEK/pMEK, anti-ERK/pERK and anti-AKT/pAKT were purchased from Biworld Antibody Company, St Louis, MO.

Western blotting

The procedure for western blotting analysis is briefly described below. After inhibiting COPS3 function by siRNA, total protein was extracted from the indicated cells with lysis buffer (0.15 M NaCl, 5 mM EDTA, pH 8, 10 mM Tris-Cl, pH 7.4, 1% Triton-X100). Protein concentrations were determined by Bradford assay. Equal amounts of protein (20 µg) were electrophoresed by 10% SDS-PAGE and transferred onto a nylon membrane (Millipore Corporation, Billerica, MA), and then the membrane was probed with the appropriate antibodies.

RNAi-mediated gene knockdown

A 19-nucleotide target sequence derived from human COPS3 mRNA (NCBI Reference Sequence: NM_003653, 272–290 bp) was designed by siRNA Wizard software of the Invitrogen Corporation. The siRNA duplexes for COPS3 were synthesized by Gene-Chem (Shanghai, China). The sequences of the siRNA targeting COPS3 were the following: sense strand 5'-GCACATTCGATA TGCAACA-3' and antisense strand 5'-TGTTGCATA TCGAATGTGC-3'. A scrambled sequence-control siRNA was also designed: sense strand 5'-TTCTCC GAACGTGTCACGT-3' and antisense strand 5'-ACGT GACACGTTCCGGAGAA-3'. Basic Local Alignment

Search Tool searches of the human genome database were carried out to ensure that the sequences would not target other gene transcripts. Plasmids expressing siRNA were under the control of the human U6 promoter in the pGCsi-U6/Neo/GFP plasmid (Gene-Chem).

Proliferation assay

Proliferation was investigated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Cells were seeded in 96-well plate in 200 µl media containing 10% dialyzed fetal bovine serum at a density of 6000 cell, in the presence or absence of COPS3 or HOS cells, respectively. On the following day 1, 2, 3, 4 and 5, to evaluate the cell number, 20 µl MTT solution (5 mg ml⁻¹ in medium) was added to the cultured cells, which were further incubated for 4 h at 37°C. After removing the remaining medium, 150 µl dimethyl sulfoxide was added to each well to solubilize the precipitate. The resulting color intensity, which is proportional to the number of viable cells, was measured by a microplate reader (versa max, Molecular Devices, Sunnyvale, CA) at 490 nm.

Migration assay

Migration assays were performed using a 24-well invasion chamber system (BD Biosciences, Franklin Lakes, NJ). Cells were seeded in the upper chamber at 2×10^5 cells ml⁻¹ in 0.1 ml serum-free 1640 media. Media supplemented with 10% fetal bovine serum was placed in the bottom well in a volume of 0.8 ml (used as a chemoattractant). After overnight incubation at 37°C in an atmosphere containing 5% CO₂, migrated cells on the lower surface were stained with 0.2% hematoxylin and counted under a light microscope. All experiments were repeated at least once.

Immunohistochemistry

Paraffin-embedded human osteosarcoma tissues were fixed in freshly prepared 10% neutral buffered formalin, embedded in paraffin wax, and cut into 5 µm sections. After baking at 60°C overnight, sections were dewaxed and rehydrated. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 10 min at room temperature. After being blocked with 5% skim milk, sections were incubated with the COPS3 antibody (5 µg ml⁻¹) at 4°C overnight, followed by the incubation with the second antibody from EnVision™ kit (Dako Cytomation, Glostrup, Denmark) for 30 min at room temperature. Reaction product was visualized with diaminobenzidine (Sigma, St Louis, MO) for 5 min at room temperature. Sections were counterstained with hematoxylin. Purified IgG from normal mouse serum was used as negative control. Evaluation of COPS3 immunoreactivity was carried out independently by two experienced pathologists, without any knowledge of the clinical data. The analysis was assessed according to both the percentage of positive cells and the intensity of the cytoplasmic staining in 10 randomly chosen microscopic fields. The staining intensity was classified as following: no staining or staining observed in <10% of tumor cells

Table 1 Relationship between COPS3 immunostaining and tumor metastasis with osteogenic sarcoma

	Osteogenic sarcoma (N = 65)	
	Metastasis (n = 28)	No-metastasis (n = 37)
<i>COPS3</i> staining		
High	16	7
Low	12	30

was given a score 0; a weak staining detected in $\geq 10\%$ of tumor cells was scored as 1+; and a moderate or strong complete staining observed in $\geq 10\%$ of tumor cells was scored as 2+ or 3+, respectively. The frequency of expression was stratified into two groups—low staining (0, 1+ immunopositivity) and high staining (2+ and 3+ immunopositivity).

Statistical analysis

Data are shown as means \pm s.d. Student's *t*-test and χ^2 -test were used for statistical analyses. $P < 0.05$ values were considered significant.

Results

OGS patients with strong COPS3 expression were more likely to metastasize

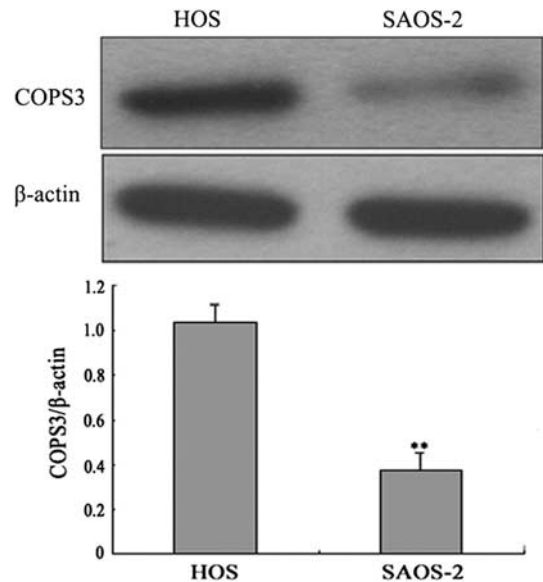
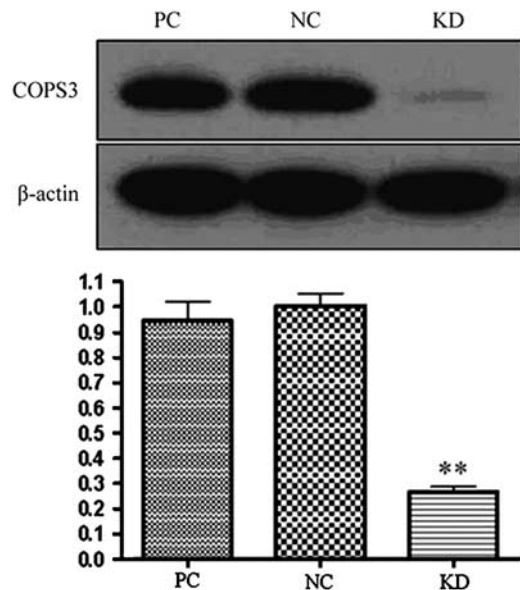
The expression of COPS3 in 65 OGS samples was analyzed immunohistochemically. Overall, 43.1% (28/65) of patients with OGS developed metastasis within 3–5 years. There was a statistically significant relationship between strong COPS3 staining and patients who developed metastatic disease, compared with those who didn't ($P < 0.01$). Overall, 18.9% (7/37) of patients without metastasis and 57.1% (16/28) of patients with metastasis showed strong staining for COPS3 (Table 1).

Cells derived from highly metastatic OGS cell lines express high levels of COPS3

We compared COPS3 expression in osteosarcoma cell lines with different metastatic potential. HOS and SAOS-2 cell lines were subjected to analysis by western blotting. A representative result from these experiments, presented in Figure 1, shows that the expression of COPS3 in SAOS-2 is about 30% compared with the expression of COPS3 in HOS.

siRNA directed against COPS3 inhibit expression of COPS3 in OGS cells

The above data from cell line and tumor tissue analyses imply a potentially important role for COPS3 in OGS. To further explore any specific role of COPS3 in OGS, the first step is to develop a cellular model that sustains forced inhibition of COPS3 expression. On the basis of this idea, we employed siRNA to silence the expression of COPS3 in HOS cell lines, which showed a high level of expression of COPS3. We employed an siRNA oligos, which can efficiently knockdown COPS3 expression in

**Figure 1** COPS3 expression in OGS cell lines with different metastatic potential. COPS3 expression is little in SAOS-2, but a high expression level in HOS.**Figure 2** COPS3 knockdown osteosarcoma model was established *in vitro*. COPS3 expression levels were measured in normal HOS, empty vector-only HOS and COPS3-knockdown HOS cells by western blots. A significantly lower level of COPS3 protein were detected in COPS3-knockdown HOS cells compared with the positive control and negative control. PC: normal HOS cell (positive control); NC: empty vector-only HOS cell (negative control); KD: COPS3-knockdown HOS cell **indicates $P < 0.01$ as compared with PC and NC, respectively.

HOS cell line. The expression of COPS3 in COPS3-silenced and non-silenced OGS cell line were confirmed at the protein levels by western blot (Figure 2), confirming that the siRNA inhibited about 77% of the expression of COPS3 in HOS cell lines.

Inhibiting COPS3 expression by siRNA inhibits proliferation and migration of HOS cells in vitro

Having established the conditions for silencing COPS3 expression in HOS cells, we used this cell line to assess the role of COPS3 in proliferation and migration, which are necessary for successful cancer metastasis. We found that silencing of COPS3 had a profound negative impact on proliferation and migration of HOS cells (~50% reduction) (Figures 3 and 4).

Inhibiting COPS3 expression by siRNA inhibits expression of the MAPK signaling pathway

After silencing COPS3 expression in HOS cell line, we examined the activity of the mitogen-activated protein kinases (MAPK) signaling pathway. Total and phosphorylated ERK1/2 and MEK were assessed. As shown

in Figure 5, the total ERK1/2 and MEK levels in HOS cells have not changed, but the phosphorylation of ERK1/2 and MEK was decreased by about 70% and 58%, respectively, after silencing of COPS3. We also examined the AKT pathway and found that neither total AKT protein levels nor phosphorylation of AKT was affected by RNAi (Figure 6).

Discussion

OGS is the most common pediatric primary bone tumor characterized by local aggressiveness and a tendency to metastasize to the lung and bone. Unfortunately, there are few clinical predictors of outcome for osteosarcoma.²⁰⁻²² Patients who present with metastases have the worst prognosis and are rarely curable.^{23,24} The mechanisms by which initiation and propagation of the metastatic process of OGS occur are largely unknown.²⁵ Therefore, prognostication and choice of therapy is largely guided by tumor grade. Clearly, identification of molecular factors that govern metastasis and/or other clinical parameter will have a significant impact on the management of patients with OGS.

Amplification of COPS3, which encodes one subunit of a highly conserved complex, the COP9 signalosome, has been reported in high-grade osteosarcoma.^{26,27} It has been reported that COPS3 has an important role in the ubiquitination and, ultimately, in the degradation of the tumor suppressor p53. The amplification and overexpression of COPS3 in osteosarcoma may represent another route to p53 protein degradation equivalent to mutational inactivation of p53.²⁸⁻³⁰ Henriksen³¹ reported that in osteosarcoma with COPS3 amplification, no cases had MDM2 amplification or p53 mutation, suggesting that COPS3 is an alternative mechanism for p53 inactivation in these tumors. The frequent amplification of COPS3

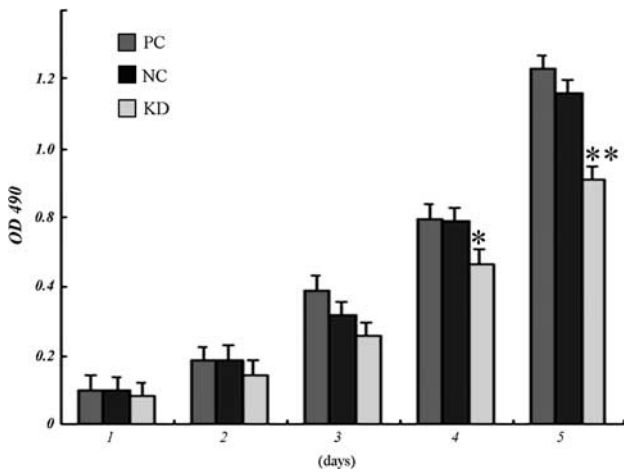


Figure 3 Proliferation assay. Our data showed that COPS3 knockdown HOS cell line was followed by a significantly reduced ability of the cells to grow, compared with PC and NC, respectively. * $P < 0.05$ and ** $P < 0.01$ as compared with PC and NC, respectively.

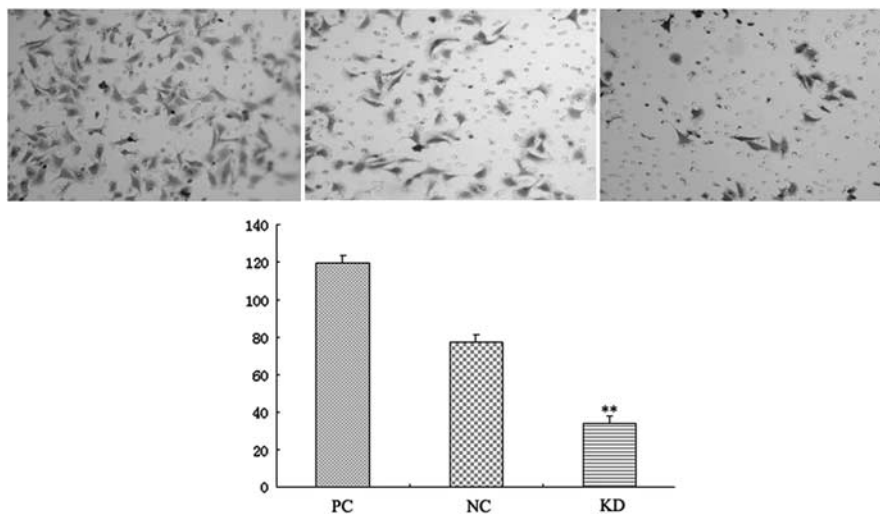


Figure 4 Migration assay. Our data showed that COPS3 knockdown HOS cell line was followed by a significantly reduced ability of the cells to migrate, compared with PC and NC, respectively. * $P < 0.05$ and ** $P < 0.01$ as compared with PC and NC, respectively.

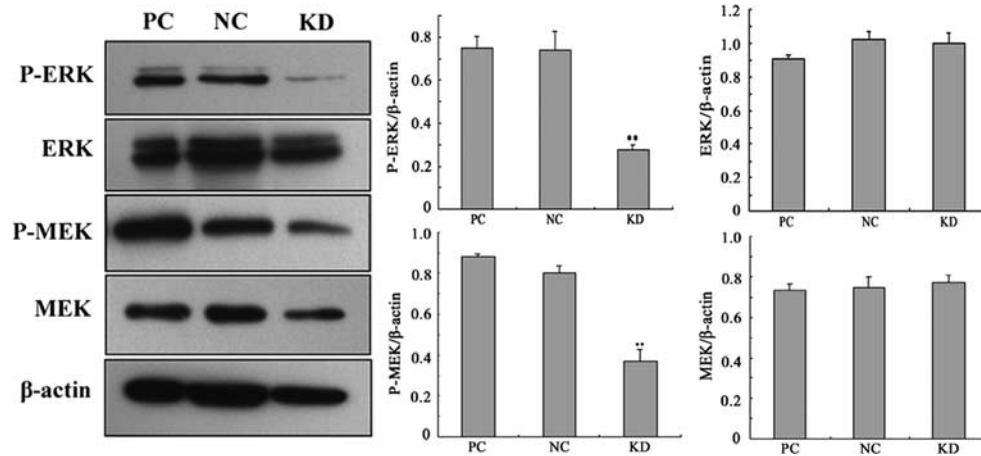


Figure 5 Expression of the MAPK signaling pathways between HOS empty vector-only HOS cell and COPS3-knockdown HOS cell. Equal amounts of total protein were resolved on SDS-PAGE gels. Western blot analysis was performed using specific antibodies against the indicated proteins. Blots were reprobed for actin to normalize each lane for protein content.

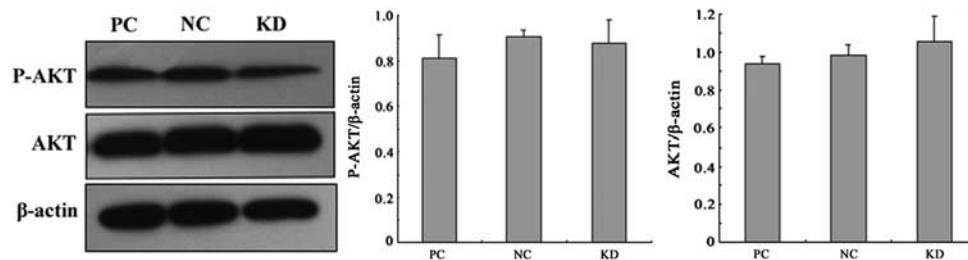


Figure 6 Changes in the expression of proteins AKT and P-AKT between HOS empty vector-only HOS cells and COPS3-knockdown HOS cells.

suggests an important role in the development or progression of osteogenic sarcoma, including regulation of the cell cycle, proliferation, differentiation, apoptosis and signal transduction.

We recently utilized quantitative real-time polymerase chain reaction to detect copy number changes for COPS3 in 155 osteosarcomas from a prospective collection of tumors, with corresponding clinical data. Results showed that osteosarcomas with COPS3 amplification and subsequent overexpression may have a growth advantage with a higher proliferative capacity, leading to increased tumor size. COPS3 amplification was significantly correlated with poor patient outcome ($P < 0.042$). In the clinical setting, we have found that tumor volume is associated with the occurrence of lung metastasis. Munajat¹⁹ reviewed 70 patients with histopathologically confirmed primary osteosarcoma in the extremities, who had magnetic resonance imaging and computed tomography of the thorax less than one month before treatment, with reference to the official report of tumor dimensions and lung metastasis by radiologists. This study found that 33 (47%) had evidence of lung metastasis. Tumor volume was directly associated with occurrence of lung metastasis ($P = 0.048$). The proportion having lung metastasis when the primary tumor volume exceeded 371 cm³ was 69%, compared with 34% of those

with smaller tumors.¹⁶ In the present study, we studied 65 osteosarcomas with corresponding clinical data and analyzed the association of COPS3 expression and metastasis. Strong COPS3 expression was detected in 23 of 65 tumors (35.3%), similar to results of previous studies.^{16,17} Overall, 18.9% (7/37) of patients without metastatic disease expressed strong staining for COPS3; however, 57.1% (16/28) of patients with metastatic disease showed strong staining for COPS3. We speculate that COPS3, acting as an oncogene, could regulate OGS growth and metastasis and thus correlate with poor patient outcome. On the basis of such a premise, we further study the role of COPS3 in the OGS cell line. We first identified COPS3 gene overexpression in highly metastatic OGS cells, compared with poorly metastatic tumor cells. We then inhibited COPS3 function by siRNA to analyze the phenotypic effects. We found that inhibition of COPS3 expression by siRNA reduced proliferation and migration of HOS by unknown mechanisms. To further evaluate this, we investigated the activity of MAPKs, which belong to serine-threonine kinases family. MAPK signaling has an important role in several features of cancer progression, including angiogenesis, proliferation, apoptosis and metastasis.³² MAPK pathway has been demonstrated to be overexpressed in most OGS and has been hypothesized to contribute to the

malignant potential of this tumor. We speculate that COPS3 may cross talk with the MAPK pathway in the development of OGS metastasis. As COPS3 is a nucleoprotein, increased expression of COPS3 may first activate the expression of MAPK pathway, leading to increased metastatic potential. Our present data show that silencing COPS3 in HOS cell inactivates the MAPK/ERK pathway by reducing the levels of phosphorylated ERK1/2 and MEK. Evidently, this concept will require evaluation through an appropriate animal model.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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