

# Cathepsin L is involved in proliferation and invasion of ovarian cancer cells

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**Abstract.** Cathepsin L (CTSL) is a lysosomal cysteine protease that has been found to be overexpressed in ovarian cancer (OC). The aim of the present study was to investigate the possible involvement of CTSL in the development of OC. In this study, RNA interference with a CTSL small hairpin RNA (CTSL-shRNA), and a plasmid carrying CTSL were used to identify the effects of this enzyme on the regulation of the malignant behavior of OC cells. OV-90 and SKOV3 human ovarian cancer cell lines were selected as cell models *in vitro* and *in vivo*. The results showed that downregulation of CTSL significantly inhibits the proliferative and invasive capability of SKOV3 cells, and that upregulation of CTSL in OV-90 cells leads to opposite effects. Compared with parental OC cells, cells in which CTSL was silenced exhibited a reduced capacity to develop into tumors in nude mice, while the growth of tumor xenografts derived from these cells was markedly constrained. In conclusion, the results suggested that CTSL contributes to the proliferation and metastasis of OC, and that CTSL may be a novel molecular target for OC treatment.

## Introduction

It is estimated that 21,980 novel ovarian cancer (OC) cases will be diagnosed in 2014 in the United States. While this number ranks OC as the ninth most common type of cancer in females, it is the fifth leading cause of cancer-related mortality in this group, with over 15,000 fatalities per year in the US (1). The five-year survival rate for early stage OC is ~92%, but it is difficult to detect OC at an early stage due to the fact that it commonly presents with vague and non-specific symptoms (2). Unfortunately, the majority of patients are diagnosed

with advanced stage disease, for which the five-year survival rate is only ~30%. Thus, early diagnosis of OC is likely to significantly improve disease outcome.

Cathepsins are lysosomal proteases of various classes. The largest group are the family of cysteine endopeptidases, which share close structural and functional characteristics with the plant cysteine endopeptidase, papain, which represents a typical example of a C1A peptidase (3). Cathepsins participate in a number of processes in healthy cells, but have also been reported to be involved in certain pathological processes, including cancer progression (4-7). In a number of cancer types, increased expression of cysteine cathepsins and alterations in their subcellular trafficking were reported (8-13).

A previous study demonstrated that CTSL, which is a member of the cathepsins family, is overexpressed in OC tissues and that its expression is inversely correlated with patient survival (14). However, it remains unclear whether inhibition of CTSL expression affects the biological function of OC cells (15). The current study aimed to analyze the effects CTSL on cultured OC cells, in terms of the growth and invasion of these cells.

## Materials and methods

**Cell lines and reagents.** The OV-90 and SKOV3 human OC cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA), and the HO-8910 and 3AO human OC cell lines were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Science (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal calf serum (Invitrogen Life Technologies, Grand Island, NY, USA), 100 U/ml penicillin, and 100 U/ml streptomycin (GE Healthcare Life Sciences). Cell culture was performed at 37°C in humidified air with 5% CO<sub>2</sub>.

**RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total cellular RNA was isolated from the four OC cell lines using TRIzol<sup>®</sup> reagent (Invitrogen Life Technologies) according to the manufacturer's instructions and quantified using a UV spectrophotometer

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(Beijing LabTech Instruments Co., Ltd., Beijing, China). RNA (2  $\mu$ g) was reverse transcribed using an access reverse transcription system (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. In brief, reaction mixtures (total volume, 20  $\mu$ l) containing 500 ng cDNA were amplified to a final concentration of 250 nM using 10  $\mu$ l of 2X Brilliant SYBR Green QPCR Master Mix kit (Agilent Technologies, Inc., Santa Clara, CA, USA). The following primers were used: Forward: 5'-GAATGGACG-GACTGCTAC-3' and reverse: 5'-CCAAAGAATACTTGCCTCA-3' (NM\_006665.5; GenBank<sup>®</sup>, International Nucleotide Sequence Database Collaboration) for CTSL and forward: 5'-TAAGAAGCTGCTGTGCTACG-3' and reverse: 5'-GACTCGTCATACTCCTGCTT-3' (NM\_001101; GenBank) for  $\beta$ -actin. Thermal cycling conditions were as follows: 94°C for 5 min and then 45 cycles at 94°C for 30 sec, followed by 60°C for 30 sec and 72°C for 45 sec. Experiments were performed in triplicate. Target genes and  $\beta$ -actin were amplified in the same reaction. The relative quantities were analyzed by comparison of  $2^{-\Delta\Delta Ct}$ .

**Vector construction and transfection.** A pcDNA3.0 vector [Cyagen Biosciences (Gunagzhou) Inc., Guangzhou, China] was used to generate pcDNA-CTSL. The CTSL small hairpin RNA (shRNA) plasmid was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA; sc-40685-SH). Vector transfection was performed according to the manufacturer's instructions, OV-90 cells were transfected with pcDNA expressing either CTSL or empty vector, and CTSL-shRNA was used to knock down the expression of CTSL in SKOV3 cells. OV-90 cells expressing either CTSL or empty vector were selected for 14 days with the antibiotic G418 following transfection. SKOV3 transfected with CTSL-shRNA was selected for 14 days with puromycin following transfection.

**Western blot analysis.** Cell samples were lysed in a lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) following collection from a 100-mm dish and lysis. Proteins (20  $\mu$ g) were resolved on a 10% SDS-PAGE gel and transferred to polyvinylidene fluoride membranes (Sigma-Aldrich, St. Louis, MO, USA). Western blot analysis was performed using a monoclonal anti-human CTSL antibody, with monoclonal anti-human  $\beta$ -actin as a control. The blocking steps and dilutions for the assessment of all proteins were conducted in 5% bovine serum albumin. Following incubation with horseradish peroxidase-conjugated antibodies (GE Healthcare Life Sciences, Chalfont, UK), labeled proteins were detected with an Enhanced Chemiluminescence-Plus detection system (GE Healthcare Life Sciences). The monoclonal anti-human CTSL antibody, anti-p38 and anti-ERK antibodies were obtained from Abcam (Cambridge, UK) and raised in rabbits. The monoclonal anti-human  $\beta$ -actin antibody was purchased from Santa Cruz Biotechnology, Inc. and raised in mouse. In addition, all secondary antibodies were monoclonal.

**Colony forming assay.** For the colony forming assay, cells were seeded evenly in 6-well plates ( $2 \times 10^2$  cells/well) and cultured for 14 days. Cells were fixed with methanol for 10 min and stained with Giemsa dye for 1 min. The number of colonies which were <1 mm was counted using a CX31 microscope

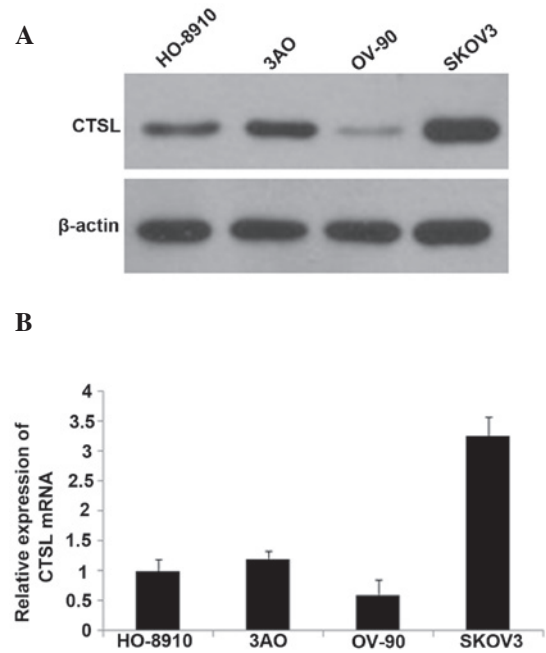


Figure 1. CTSL expression in four human OC cell lines with varying metastatic potential. (A) Representative western blot. CTSL protein expression levels in HO-8910, 3AO, OV-90 and SKOV3 cell lines were determined by western blot analysis. SKOV3 and OV-90 cell lines expressed the highest and lowest levels of CTSL protein, respectively. (B) Quantification of CTSL mRNA expression in each cell line relative to controls, as detected by reverse transcription-quantitative polymerase chain reaction. SKOV3 and OV-90 cells showed the highest and lowest expression of CTSL mRNA, respectively. The results were obtained from three independent experiments. The expression of  $\beta$ -actin protein and mRNA was used as a control. CTSL, cathepsin-L; OC, ovarian cancer.

(Olympus Corporation). Plate clone formation efficiency was calculated as follows: Clone formation efficiency (%) = number of colonies/number of cells inoculated. Each experiment was performed in triplicate.

**3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide reduction (MTT) assay.** Cells were seeded into 96-well plates at a density of 2,000 cells/well. There were four samples from each group. Cells were incubated with 0.2% MTT for 4 h at 37°C, 100  $\mu$ l dimethylsulfoxide was added to each well to dissolve the crystals and cells were counted daily by reading the absorbance at 490 nm using a microplate reader (Multiskan MK3; Thermo Fisher Scientific, Waltham, MA, USA).

**Cell invasion assay.** The upper chambers of a 24-well transwell plate (Corning Incorporated, Corning, NY, USA) were coated with 50% Matrigel (BD Biosciences Franklin Lakes, NJ, USA) in phosphate-buffered saline. Cells were incubated in the upper chamber. Following 24 h incubation, invaded cells were stained with 0.5% crystal violet, examined by bright field microscopy (CX31; Olympus Corporation, Tokyo, Japan) and photographed (DP21 Camera; Olympus Corporation). The invasion rate was quantified by counting the invaded cells in five randomly selected fields per chamber under a fluorescence microscope (IX71; Olympus Corporation). Three independent experiments were conducted for each group.

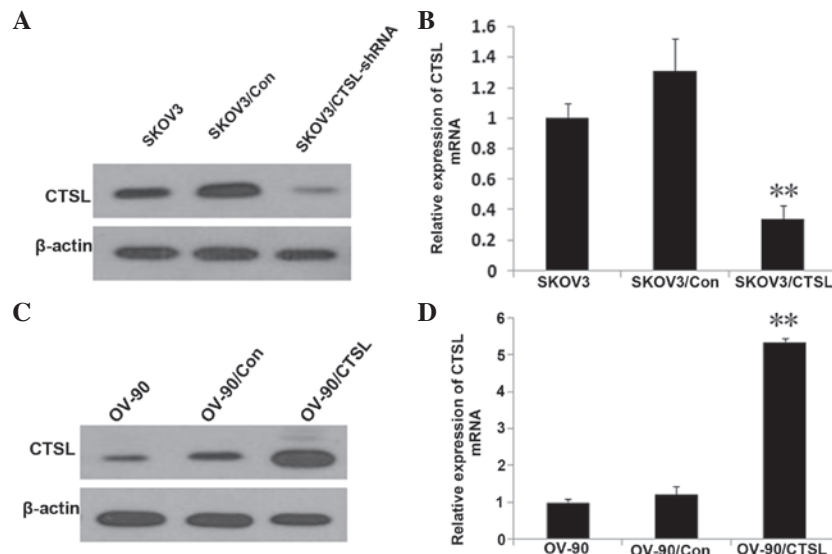


Figure 2. (A) Effect of CTSL specific shRNAs on the levels of CTSL protein in SKOV3 cells. Levels of CTSL protein were assessed by western blotting. (B) Effect of CTSL specific shRNAs on the levels of CTSL mRNA in SKOV3 cells. Levels of CTSL protein were assessed by reverse transcription-quantitative polymerase chain reaction. (C) CTSL protein levels in OV-90 cells stably transfected with pcDNA-CTSL, OV-90/Con cells and OV-90 cells. (D) CTSL mRNA levels in OV-90 cells stably transfected with pcDNA-CTSL, OV-90/Con cells and OV-90 cells. The expression of  $\beta$ -actin mRNA and protein was used as a control. \*\* $P < 0.01$ . CTSL, cathepsin L; shRNA, small hairpin RNA; SKOV3/Con, SKOV3 cells transfected with control shRNA, SKOV3/CTSL-shRNA, SKOV3 cells transfected with CTSL shRNA; OV-90/Con, OV-90 cells transfected with empty vector; OV-90/CTSL, OV-90 cells transfected with CTSL vector.

**Tumorigenicity assay.** Female/male athymic BALB/C nu/nu mice (4- to 6-week old) were purchased from the Central Laboratory of Animal Science at Southern Medical University (Guangzhou, China) and maintained in laminar flow cabinets under specific pathogen-free conditions. SKOV3/shCTSL and SKOV3/Con ( $5 \times 10^6$  cells) were injected subcutaneously into the flanks of the nude mice. Tumor growth was evaluated every 7 days for 3 weeks following inoculation. Tumor volume (V) was determined by measuring the largest (a) and the smallest (b) axis using calipers, and calculated as  $V = 0.5ab^2$ . Mice handling and experimental procedures followed institutional guidelines. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of the 458<sup>th</sup> Hospital of PLA (Guangzhou, China).

**Statistical analysis.** Unless otherwise stated, all data are presented as the mean  $\pm$  standard error of the mean. Statistical significance was determined by a t-test or analysis of variance followed by assessment of differences using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference

**Results**

**Expression of CTSL in four OC cell lines.** To investigate the expression of CTSL in nasopharyngeal carcinoma cells, RT-qPCR and western blotting was conducted in HO-8910, 3AO, OV-90 and SKOV3 human OC cell lines. The mRNA and protein levels of CTSL in the three cell lines are shown in Fig. 1. The results showed that SKOV3 and OV-90 expressed the highest and lowest levels of CTSL mRNA and protein, respectively. Thus, these two cell lines were selected for subsequent investigation into the biological function of CTSL in OC.

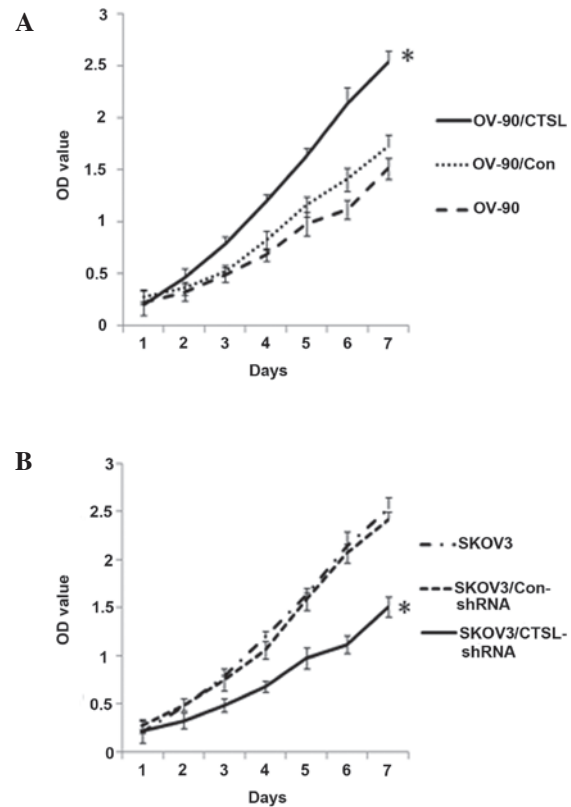


Figure 3. Effect of CTSL on cell proliferation *in vitro*. (A) MTT assay of OV-90 cells overexpressing CTSL. The number of viable cells was assessed using an MTT assay at 1, 2, 3, 4, 5, 6 and 7 days. Each sample was tested in triplicate and the results are reported as optical density readings. (B) MTT assay of SKOV3 cells with downregulation of CTSL. Values represent the mean  $\pm$  standard deviation of at least three independent experiments. \* $P < 0.05$ . CTSL, cathepsin L; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bormide reduction; OV-90/CTSL, OV-90 cells transfected with CTSL vector; OV-90/Con, OV-90 cells transfected with empty vector; SKOV3/Con-shRNA, SKOV3 cells transfected with control shRNA; SKOV3/CTSL-shRNA, SKOV3 cells transfected with CTSL shRNA; shRNA, small hairpin RNA.

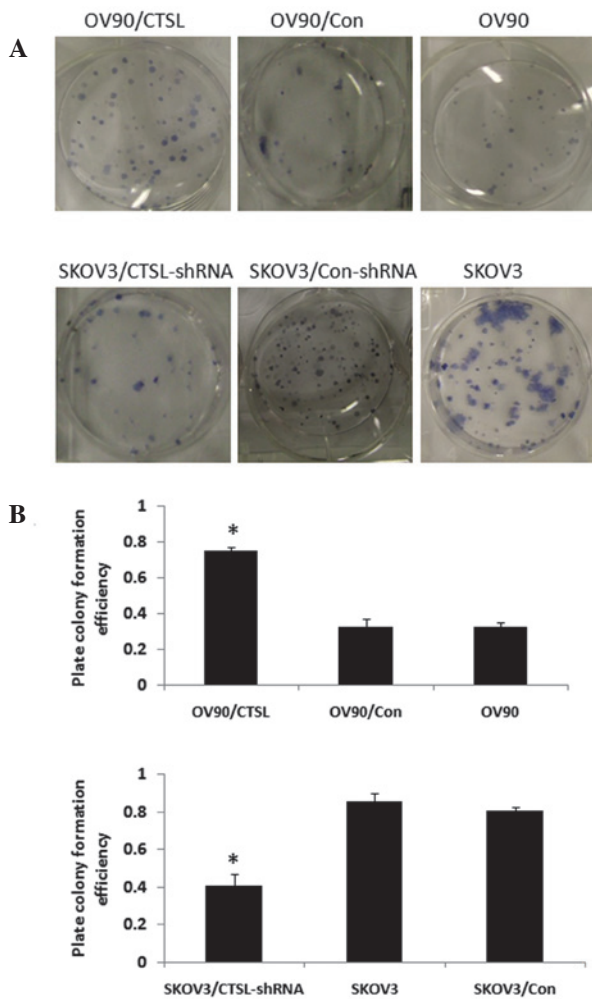


Figure 4. Effect of CTSL gene on liquid colony formation potential *in vitro*. (A) Following 12 days of culture, cells were stained with May-Giemsa and those <1 mm were counted. (B) Quantification of colony-forming ability in each group. Data are presented as the mean  $\pm$  standard deviation from at least three independent experiments. \* $P < 0.05$ . CTSL, cathepsin L; OV90/CTSL, OV-90 cells transfected with CTSL vector; OV90/Con, OV-90 cells transfected with empty vector; SKOV3/CTSL-shRNA, SKOV3 cells transfected with CTSL shRNA; SKOV3/Con, SKOV3 cells transfected with control shRNA; shRNA, small hairpin RNA.

*Vector stably expressing CTSL-shRNA causes effective and specific downregulation of cathepsin L expression.* The knock down efficiencies of three CTSL specific shRNAs in SKOV3 cells were evaluated using RT-qPCR. Relative CTSL mRNA levels in individual stably transfected cells were normalized against mRNA levels of a reference gene,  $\beta$ -actin, which were measured in the same run. As shown in Fig. 2A and B, cells transfected with CTSL-shRNA had a significantly reduced level of CTSL protein compared with that of parental SKOV3 cells and Con-transfected cells. The above results demonstrated that expression of CTSL was downregulated specifically and effectively using specific CTSL shRNA. SKOV3 cells transfected with CTSL-shRNA were termed SKOV3/shCTSL, and SKOV3 cells transfected with Con were termed SKOV3/Con.

*CTSL overexpression in OC cells.* OV-90 cells transfected with pc.DNA3.0-CTSL plasmid exhibited a significant

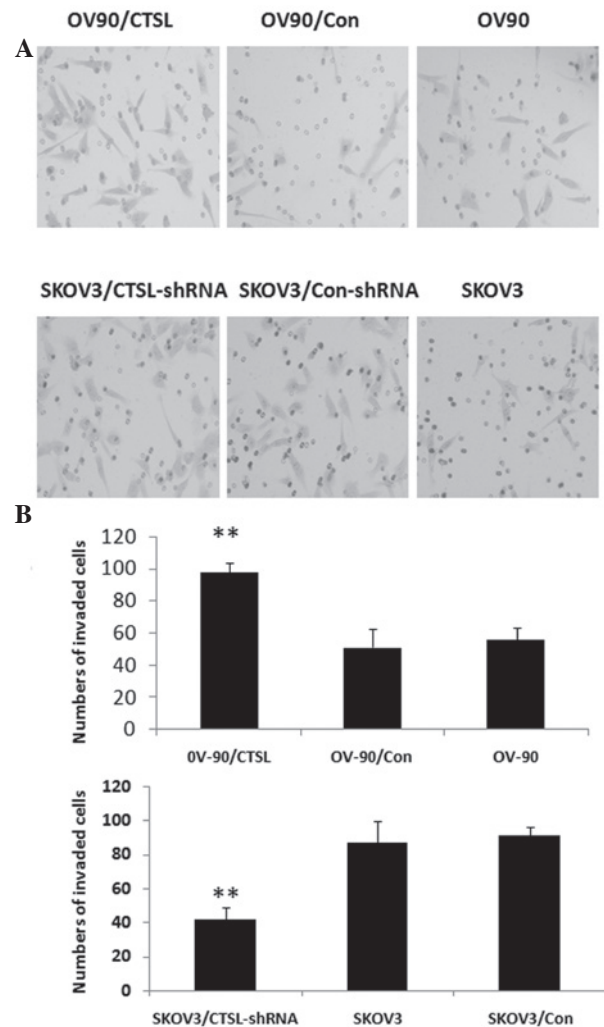


Figure 5. Effect of CTSL on the invasive potential of OC cells. (A) *In vitro* transwell invasion assay were conducted to compare and quantify the invasive capacity of OC cells. (B) Quantification of transwell migration assay. The data are presented as the mean  $\pm$  standard deviation of three independent experiments. Magnification,  $\times 200$ . \*\* $P < 0.01$ . CTSL, cathepsin L; OC, ovarian cancer; OV-90/CTSL, OV-90 cells transfected with CTSL vector; OV-90/Con, OV-90 cells transfected with empty vector; SKOV3/CTSL-shRNA, SKOV3 cells transfected with CTSL shRNA; SKOV3/Con, SKOV3 cells transfected with control shRNA; shRNA, small hairpin RNA.

increase in the expression level of CTSL compared with the vector control group (Fig. 2C and D). The overexpression of CTSL was confirmed by western blot analysis and RT-qPCR. The OV-90 cells overexpressing CTSL were termed OV-90/CTSL.

*Effect of CTSL on cell proliferation of OC cells.* The proliferative activity of tumor cells is important in the invasion and metastasis of tumors. Having established SKOV3/shRNA cells and OV-90/CTSL cells, cell proliferation activity of the transfected cells was measured using an MTT assay. As shown in Fig. 3A and B, the growth of SKOV3 cells *in vitro* was markedly inhibited following the transfection of CTSL-shRNA ( $P < 0.05$ ), whereas overexpression of CTSL promoted OV-90 cell proliferation ( $P < 0.05$ ). This indicates a positive correlation between the expression of CTSL and the rate of OC cell growth.

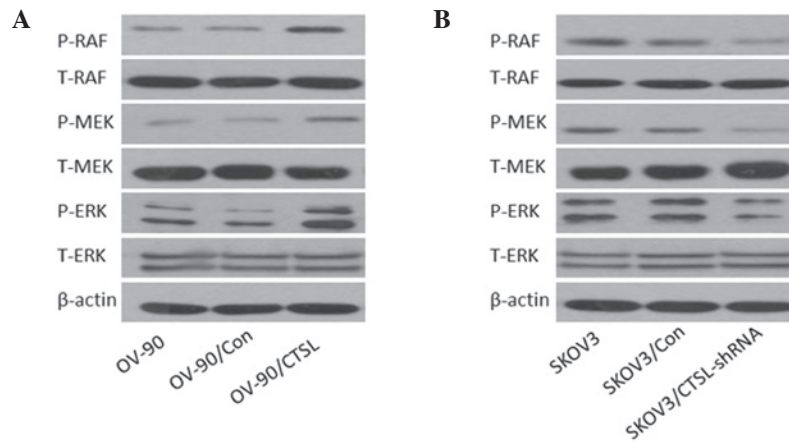


Figure 6. Effect of CTSL on the Raf/MEK/ERK pathway. Total cellular protein was collected and subjected to western blot analysis. Stable overexpression of CTSL increased the level of P-RAF, P-MEK and P-ERK expression. Downregulation of CTSL led to opposite effects. CTSL, cathepsin L; ERK, extracellular signal-regulated kinase; P-, phosphorylated; T-, total; OV-90/Con, OV-90 cells transfected with empty vector; OV-90/CTSL, OV-90 cells transfected with CTSL vector; SKOV3/Con, SKOV3 cells transfected with control shRNA; SKOV3/CTSL-shRNA, SKOV3 cells transfected with CTSL shRNA; shRNA, short hairpin RNA.

**Effect of CTSL on the colony forming potential of OC cells.** In order to examine the ability of SKOV3/CTSL-shRNA cells and OV-90/CTSL to form colonies, single-cell suspensions were plated at a density of 100 cells in 30-mm culture dishes. As shown in Fig. 4, following 12 days of culture, SKOV3/shRNA cells, compared with parental SKOV3 and SKOV3/Con cells, had a significant reduction in the ability to form colonies ( $P < 0.05$ ), whereas OV-90/CTSL showed an increase in this ability compared with parental OV-90 and OV-90/Con cells ( $P < 0.05$ ).

**Effect of CTSL on invasiveness of OC cells.** OV-90/CTSL cells showed higher levels of invasiveness than either parental OV-90 or OV-90/Con cells ( $P < 0.01$ , respectively; Fig. 5A). The percentage of invading cells in the OV-90/CTSL, OV-90 and OV-90/Con groups was 100, 95 and 37.4% respectively. The results indicate that CTSL is associated with the invasiveness of OC cells. In addition, SKOV3/shRNA cells displayed a significant reduction in invasion ability compared with SKOV3/Con or parental SKOV3 cells ( $P < 0.01$ ). As shown in Fig. 5B, the percentage of migrating cells in the SKOV3/shRNA group was 45.9%, while in the SKOV3 and SKOV3/Con groups it was 98 and 100%, respectively. Inhibition of CTSL caused significantly attenuated migration in SKOV3 cells.

**Effects of CTSL on expression of mitogen-activated protein kinase (MAPK) transduction.** As shown in Fig. 6, western blotting was conducted to detect the expression of components of MAPK transduction in OC cell lines. Inhibition of CTSL resulted in significant reduction in the expression of phosphorylated MEK, RAF and extracellular signal-regulated kinase (ERK) (p-MEK, p-RAF and p-ERK, respectively) in SKOV3 cells, whereas overexpression of CTSL resulted in a significant increase in p-MEK, p-RAF and p-ERK expression in OV-90 cells. Protein levels of p-MEK, p-RAF and p-ERK in SKOV3/shRNA were lower than those in the SKOV3 and SKOV3/Con groups, and in the OV-90/CTSL groups these levels were higher than those in the OV-90 and OV-90/Con groups.

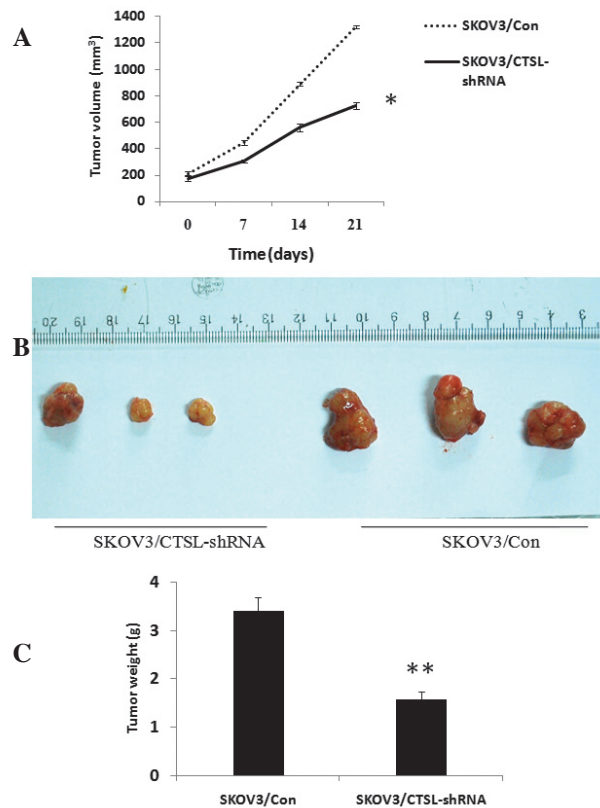


Figure 7. Effect of knockdown of CTSL on the tumorigenicity of SKOV3 cells in nude mice. SKOV3/CTSL-shRNA and SKOV3/Con cells were injected subcutaneously into nude mice (A) Growth of tumors over a 21 day period in each group. \* $P < 0.05$ . (B) Representative images of tumors removed and weighed at day 21 ( $n = 3$ ). (C) Average tumor weights ( $n = 4$ ) of control and CTSL-silenced groups were  $1.58 \pm 0.23$  and  $3.41 \pm 0.15$  g, respectively. \*\* $P < 0.01$ . CTSL, cathepsin L; SKOV3/CTSL-shRNA, SKOV3 cells transfected with CTSL shRNA; SKOV3/Con, SKOV3 cells transfected with control shRNA; shRNA, small hairpin RNA.

**CTSL gene silencing suppresses cell proliferation in vivo.** As shown in Fig. 7, the effect of CTSL on *in vivo* tumor growth was assessed by subcutaneous injection of SKOV3/shRNA

and SKOV3/Con cells into nude mice for a period of 21 days. A marked reduction in tumor size in the SKOV3/shRNA group was observed compared with that in the control group ( $P < 0.05$ ). On day 21 following cell injection, the average tumor weight ( $n = 4$ ) of the SKOV3/shRNA and SKOV3/Con groups was  $1.58 \pm 0.23$  and  $3.41 \pm 0.15$  g, respectively ( $P < 0.01$ ), indicating that knockdown of CTSL in OC cells reduced their tumorigenic potential.

## Discussion

The present study showed that overexpression of CTSL is important in the progression of OC. These findings suggest that CTSL is a significant contributor to the proliferation, invasion and migration of OC cells.

Cells require interactions with extracellular matrix (ECM) components in order to undergo normal morphogenesis with respect to organogenesis. ECM is involved in regulating cell shape and numerous cellular functions, including adhesion, migration, proliferation, polarity, differentiation and apoptosis. In pathological conditions, such as cancer, the increased synthesis of certain ECM components, in addition to the increased breakdown of a number of these molecules, with consequent generation of ECM cleavage products, can contribute to cancer growth and progression. A number of growth factors, including fibroblast growth factor and vascular endothelial growth factor are stored in the ECM environment and are released upon protease-dependent cleavage of ECM components, which further indicates the importance of the ECM in the regulation of cellular functions. Tumor metastasis remains the principal cause of treatment failure and poor prognosis in patients with OC. Metastasis is a multistage process, which includes the proteolysis, motility and migration of cells, proliferation at a new site, and neoangiogenesis. A crucial step in the process of intra- and extravasation of cancer cells is the activation of proteolytic enzymes capable of degrading the ECM. CTSL degrades ECM and basement membrane components, and this enzyme is involved in tumor metastasis and angiogenesis. Elevated expression of CTSL mRNA and protein in tumors has been demonstrated in tissue specimens derived from gliomas (16), oral squamous cell carcinoma (13), hepatocellular carcinoma (17), prostate carcinoma (18), bladder carcinoma (19), colon carcinoma (20), stomach carcinoma (21), breast carcinoma (22), pancreatic adenocarcinoma (23), esophageal carcinoma (24) and chronic myeloid leukemia (25). Notably, increased CTSL levels were often found to be associated with a reduction in patient survival rate, increased carcinoma metastasis and high microvessel density.

Regardless of the potential significance of CTSL in OC, the functional role of CTSL in this disease has not been clearly defined and evidence of its oncogenic activity in OC is lacking. To investigate the role of CTSL in OC, endogenous CTSL expression in an OC cell line (SKOV3) was silenced using shRNA. Properties of the CTSL-depleted cells were then analyzed and compared with control cells in various functional assays. The results showed that CTSL knockdown led to reduced cell proliferation and anchorage-independent growth. Furthermore, the motility and invasiveness of cells were significantly impeded with CTSL depletion. In addition, overexpression of CTSL promoted these characteristics of

OV-90 cells. To the best of our knowledge, the current study provides the first validation of the effect of the functional loss of CTSL expression *in vivo*. SKOV3 cells with high levels of CTSL expression displayed an increased ability to form tumors in nude mice. These results affirmed the findings from the other experiments conducted in this study, that CTSL exerts an oncogenic effect on SKOV3 cells. The results suggest a possible explanation for the poorer outcomes observed in OC patients with CTSL overexpression.

The MAPK signaling pathway is a major determinant in the control of diverse cellular processes, such as proliferation, survival, differentiation and motility, and is associated with tumor development (26). The results from the present study indicate that the antiproliferative effects of CTSL in OC cells are partially mediated by MAPK signaling. Suppression of CTSL in OC cells inhibited cell proliferation in association with a decrease in phosphorylation of MEK, RAF and ERK. Furthermore, overexpression of CTSL enhanced the growth of OV-90 cells by increasing phosphorylation of MEK, RAF and ERK. However, it was also observed that even following knockdown of CTSL, OC cells nonetheless possessed capabilities of invasion and metastasis. It is postulated that other factors, including urokinase and matrix metalloproteinases, also influence these characteristics of OC (27,28), which requires further investigation.

In the present study, downregulation of CTSL by shRNA was shown to inhibit OC cell growth and migration, and overexpression of CTSL by cDNA transfection was demonstrated to promote OC cell proliferation and motility. Previous studies have shown that interference with CTSL expression additionally regulates at least two major signaling pathways (Wnt/ $\beta$ -catenin and transforming growth factor- $\beta$ ) in cancers. It is therefore possible that interfering with CTSL expression and function exerts broad-spectrum biological effects that may be beneficial for the treatment of OC. Further studies of the mechanisms by which CTSL regulates multiple signaling pathways may reveal other points of intervention for this important target.

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