Original Article

Cathepsin L upregulation-induced EMT phenotype is associated with the acquisition of cisplatin or paclitaxel resistance in A549 cells

Mei-ling HAN¹, #, Yi-fan ZHAO¹, #, Cai-hong TAN¹, ², Ya-jie XIONG¹, Wen-juan WANG¹, Feng WU³, Yao FEI¹, Long WANG³, Zhong-qin LIANG¹,*

¹Department of Pharmacology, College of Pharmaceutical Sciences, Soochow University, Suzhou 215123, China; ²Department of Pharmacy, Affiliated Hospital of Jiangsu University, Zhenjiang 212001, China; ³Department of Pharmacology, School of Pharmacy, Nantong University, Nantong 226001, China

Aim: Cathepsin L (CTSL), a lysosomal acid cysteine protease, is known to play important roles in tumor metastasis and chemotherapy resistance. In this study we investigated the molecular mechanisms underlying the regulation of chemoresistance by CTSL in human lung cancer cells.

Methods: Human lung cancer A549 cells, A549/PTX (paclitaxel-resistant) cells and A549/DDP (cisplatin-resistant) cells were tested. The resistance to cisplatin or paclitaxel was detected using MTT and the colony-formation assays. Actin remodeling was observed with FITC-Phalloidin fluorescent staining or immunofluorescence. A wound-healing assay or Transwell assay was used to assess the migration or invasion ability. The expression of CTSL and epithelial and mesenchymal markers was analyzed with Western blotting and immunofluorescence. The expression of EMT-associated transcription factors was measured with Western blotting or q-PCR. BALB/c nude mice were implanted subcutaneously with A549 cells overexpressing CTSL, and the mice were administered paclitaxel (10, 15 mg/kg, ip) every 3 d for 5 times.

Results: Cisplatin or paclitaxel treatment (10–80 ng/mL) induced CTSL expression in A549 cells. CTSL levels were much higher in A549/PTX and A549/DDP cells than in A549 cells. Silencing of CTSL reversed the chemoresistance in A549/DDP and A549/TAX cells, whereas overexpression of CTSL attenuated the sensitivity of A549 cells to cisplatin or paclitaxel. Furthermore, A549/DDP and A549/TAX cells underwent morphological and cytoskeletal changes with increased cell invasion and migration abilities, accompanied by decreased expression of epithelial markers (E-cadherin and cytokeratin-18) and increased expression of mesenchymal markers (N-cadherin and vimentin), as well as upregulation of EMT-associated transcription factors Snail, Slug, ZEB1 and ZEB2. Silencing of CTSL reversed EMT in A549/DDP and A549/TAX cells; In contrast, overexpression of CTSL induced EMT in A549 cells. In xenograft nude mouse model, the mice implanted with A549 cells overexpressing CTSL exhibited significantly reduced sensitivity to paclitaxel treatment, and increased expression of EMT-associated proteins and transcription factors in tumor tissues.

Conclusion: Cisplatin and paclitaxel resistance is associated with CTSL upregulation-induced EMT in A549 cells. Thus, CTSL-mediated EMT may be exploited as a target to enhance the efficacy of cisplatin or paclitaxel against lung cancer and other types of malignancies.

Keywords: human lung cancer; cisplatin; paclitaxel; chemotherapy resistance; cathepsin L; EMT; A549 cells; xenograft nude mouse model

Acta Pharmacologica Sinica (2016) 37: 1606–1622; doi: 10.1038/aps.2016.93; published online 14 Nov 2016

Introduction
To date, lung cancer presents the highest morbidity among all types of malignancies. Up to 30% of all cancer-related deaths are attributed to lung cancer because of its high mortality rate[1]. Current treatment strategies include surgical resection, chemotherapy, radiation therapy, targeted therapy, or a combination of treatments, depending on the disease type and stage[2, 3]. Currently, chemotherapy remains the main method for lung cancer treatment.

Cisplatin and paclitaxel are the most common drugs used to treat non-small-cell lung cancer (NSCLC). Paclitaxel is an important first-line chemotherapeutic agent used to treat a wide range of malignancies; this drug targets the microtubule...
cytoskeleton, which is important in cell division\(^\text{4-8}\). Cisplatin, a platinum chemotherapeutic agent, is a major frontline drug used to treat various human malignant tumors, including lung carcinoma\(^\text{9, 10}\). However, the clinical usage of these drugs is limited because of intrinsic and acquired resistance\(^\text{11, 12}\). Thus, the discovery of a key molecule associated with tumor drug resistance and metastasis would provide an important target for the treatment of lung cancer.

At present, mechanisms promoting or enabling drug resistance include drug inactivation, drug target alteration, drug efflux, DNA damage repair, and cell death inhibition. The role of epithelial-to-mesenchymal transition (EMT) in cancer drug resistance is an emerging area in research\(^\text{13, 14}\). EMT is a unique process of molecular reprogramming and phenotypic changes characterized by a transition from polarized immortal epithelial cells to motile mesenchymal cells\(^\text{15}\); thus, these cells undergo changes in shape and cytoskeletal organization and acquire mesenchymal characteristics that are important for metastasis\(^\text{16, 17}\). Cancer cells undergoing EMT have been found to show increased resistance to apoptosis and chemotherapeutic drugs\(^\text{18}\). EMT is directly controlled by numerous extracellular signals and pathways\(^\text{19}\). The blockade of these signaling pathways is critical for reversing EMT and its related biological effects, including drug sensitivity.

Cathepsin L (CTSL) is a cysteine protease that belongs to the papain-like family (peptidase C1A), which has been reported to be associated with tumor occurrence, development, and metastasis\(^\text{20-22}\). CTSL plays an important role in the degradation and renewal of intracellular proteins; it is also involved in many essential physiological processes, including the activation of prohormones and the presentation of antigens, as well as organ development\(^\text{23-25}\). In recent years, CTSL was reported to be associated with drug resistance. Zheng et al suggested that CTSL inhibition in drug-resistant cells facilitates the induction of senescence and the reversal of drug resistance\(^\text{26}\), and CTSL inhibition-mediated drug target stabilization may be used as an alternative approach to enhance the efficacy of chemotherapy\(^\text{27}\). Nevertheless, the roles and mechanisms by which CTSL regulates drug resistance remain to be further elucidated.

Based on the above knowledge, we hypothesized that CTSL and EMT may be involved in drug resistance. In the present study, we demonstrated that CTSL is a regulator of drug resistance in A549 cells, and the regulation of chemoresistance by CTSL is mediated through its effects on the expression of EMT-associated transcription factors, which are inducers of EMT. Thus, we assumed that CTSL may represent a novel therapeutic target to reinforce the efficacy of cancer chemotherapy.

**Materials and methods**

**Materials**

Cell culture reagents and Lipofectamine reagent were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Phalloidin was obtained from Sigma-Aldrich (St Louis, MO, USA). The antibodies used in this study were anti-N-cadherin, anti-E-cadherin, and anti-Snail (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA); anti-cathepsin L (Abcam); anti-β-actin (MultiSciences Biotech, Hangzhou, China); and anti-Slug (Cell Signaling Technology, Danvers, MA, USA).

**Cell lines and culture**

The human lung cancer A549 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. A549 cells were obtained from a 58-year-old white male with poorly differentiated lung adenocarcinoma. A549/PTX and A549/DDP cells were purchased from Shanghai MEIXUAN Biological Science and Technology Co, Ltd. A549/PTX and A549/DDP cells were cultured in RPMI-1640, and A549 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/mL)/streptomycin (100 U/mL) at 37°C in a humidified atmosphere with 5% CO\(_2\).

**Cytotoxicity assay**

Methyl thiazolyl tetrazolium (MTT) was used to measure the viability and proliferation of cells. Cells were seeded into 96-well plates at a density of 10\(^4\) cells per well. The cells were then cultured for 24 h in 100 μL of RPMI-1640 or DMEM complete medium. After pretreatment with different concentrations of paclitaxel or cisplatin for 48 h, 10 μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h at 37°C, and 100 μL of 1% acid was added to each well to dissolve the blue formazan crystals. The optical density was measured at 570 nm. All assays were performed in triplicate.

**siRNA transfection**

CTSL siRNA and control siRNA were obtained from GenePharma (Shanghai, China). For transfection, siRNA was mixed with Lipofectamine® 3000 Reagent (Invitrogen) and then transfected into A549/DDP and A549/TAX cells. After 6 h, the supernatant was replaced with fresh medium containing 10% FBS and cultured for another 24 h. A total of four siRNA sequences was used for transfection (Table 1).

| siRNA       | Base sequence                  |
|-------------|--------------------------------|
| Negative control (NC) Sense | 5'-UUUCUGCAGCGUCAGCGUTT-3' |
|             | Anti-sense                    | 5'-CCGUGCACGUCAGCCGAATT-3' |
| CTS-homo-391 (1) Sense | 5'-GCCUCAGCUCUCUACAUAGTT-3' |
|             | Anti-sense                    | 5'-AUGCUUGAAGACUGAGGCTT-3' |
| CTS-homo-449 (2) Sense | 5'-CGAUGCGCAACAGAGUAUATT-3' |
|             | Anti-sense                    | 5'-UAUAAUCUGUUGUGCAUGTT-3' |
| CTS-homo-994 (3) Sense | 5'-CCGAGAUGCUGCUUGCUAATT-3' |
|             | Anti-sense                    | 5'-UUAGCCACAGAAUACUGGTT-3' |
| CTS-homo-1112 (4) Sense | 5'-CCUUCUGCUGUUCUAAAGATT-3' |
|             | Anti-sense                    | 5'-UCUUAUAAGAAGCAAGGGTT-3' |

**Table 1. siRNA sequences.**
Lentivirus transfection
A549 cells were seeded in six-well plates in DMEM culture medium supplemented with 10% FBS. At 24 h after seeding, the cells were treated with 2×10⁵ titration units of lentivirus packaging and synthesis by GeneChem (Shanghai, China). Polybrene was added at a concentration of 5 ng/μL to enhance the efficiency of viral infection. The cells were harvested 4 d after infection for either RNA extraction or protein lysate preparation.

Wound-healing assay
For the wound-healing assay, the cells were grown in six-well plates. When confluency was achieved, the cells were scratched using a pipette tip, rinsed to remove debris, and then further incubated with fresh culture medium containing 1% FBS for 24 h. Cell migration images were captured at 0 and 24 h. The wound-healing index, which was determined as a percentage, was quantitatively analyzed using 20 randomly selected distances across the wound at 0 and 24 h, divided by the distance measured at 0 h.

Transwell invasion assay
The invasion assay was performed using 24-well Matrigel invasion chambers (BD Biosciences). The cells were trypsinized and reseeded in the upper chamber at a concentration of 1×10⁶/mL in 200 μL of RPMI-1640 free with FBS. The lower chamber contained 800 μL of RPMI-1640 supplemented with 10% FBS. After 24 h, the cells on the upper surface of the filters were removed, and the cells on the lower surface were fixed with methanol and stained with crystal violet.

Actin staining of the cytoskeleton
Cells were grown on coverslips and fixed with 4% fresh paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% TritonX-100 in PBS for 20 min, and then blocked with 5% bovine serum albumin (BSA) at room temperature for 1 h. Subsequently, the cells were stained with FITC-Phalloidin for 2 h at room temperature in the dark. After washing, the cells were counterstained with DAPI for 10 min. Confocal microscopy (Carl Zeiss, LSM 710) was employed to observe the distribution of F-actin.

Western blot analysis
Cells were harvested using a plastic scraper and then washed twice with cold PBS. Afterward, the cells were homogenized in lysis buffer. Proteins in the lysates were quantified using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The lysates were loaded and separated on 10% or 3% SDS-PAGE gels, and the proteins were transferred onto nitrocellulose blotting membranes. The membranes were blocked with 5% BSA for 1 h and then incubated with primary antibodies overnight. After washing three times, the blots were incubated with secondary antibodies for 1 h. The immunoblots were detected using the Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE, USA).

Quantitative q-PCR analyses of ZEB1 and ZEB2 amplifications
q-PCR was performed on an ABI7500 thermocycler with 7500 software v2.03 (Life Technologies Corporation). The ZEB1 and ZEB2 primer sequences (synthesized by Shanghai Sangon Biotechnology Co, Ltd) were as follows: ZEB1 forward primer, 5’-GAAAATGACAAAAACCATGATCTCA-3’; ZEB1 reverse primer, 5’-CAGGTGCCCTAGGAAATTGA-3’; ZEB2 forward primer, 5’-TTCCATTGCTGTGGGCTT-3’; ZEB2 reverse primer, 5’-TTGTGGAGGGGTTACTGTTGG-3’.

Immunofluorescence staining
The day after seeding on coverslips, the cells were treated as required for the experiment. The cells were fixed with methanol for 10 min at 4°C and permeabilized for 10 min with 0.1% Triton X-100. The cells were then incubated for 1 h in blocking buffer (1% BSA and 0.1% Triton X-100) at 4°C. For immunofluorescence (IF), the cells were incubated with antibodies against E-cadherin (Santa Cruz), N-cadherin (Santa Cruz), and vimentin (Abcam) at 4°C overnight. The cells were then rinsed three times with PBS and incubated with the appropriate biotinylated secondary antibodies for 1 h. Alexa Fluor 488 (Molecular Probes, 1:500) and Alexa Fluor 594 goat anti-mouse (Molecular Probes, 1:500) antibodies were used as tertiary antibodies for 1 h. The cells were counterstained with 0.5 ng/mL DAPI for 15 min at room temperature. Coverslips were mounted on slides with VECTASHIELD Mounting Medium for fluorescence and analyzed by confocal microscopy.

Animal experiments
This study was carried out in accordance with the principles of the Declaration of Helsinki and approved by the Ethics Committee of Soochow University Medical School. Five-week-old male nude (BALB/c) mice (Animal Experiment Center of Soochow University, Suzhou, China) were used in the experiments. A549-Vector and A549-Over-CTSL cells (5×10⁶ cells/0.1 mL medium/mouse) were injected into the mice subcutaneously to generate the mouse models. Xenografts were allowed to grow to approximately 100 mm³ over 2 weeks and randomly divided into six groups (n=5 in each group) as follows: vector-control (100 μL, saline solution), vector-PTX-low (10 mg/kg), vector-PTX-high (15 mg/kg), over-CTSL-control (100 μL, saline solution), over-CTSL-PTX-low (10 mg/kg), and over-CTSL-PTX-high (15 mg/kg). Paclitaxel was administered by intraperitoneal injection every 3 d for 2 weeks. From the day of intervention, the longest diameter (a) and the shortest diameter (b) of the tumor were measured using digital calipers every 3 d, and the tumor volume (V=π/6ab²) was calculated. At the end of the experiments, the tumors were subcutaneously harvested following animal sacrifice by cervical dislocation. Tumor tissues were harvested from these mice for immunohistochemistry or Western blot analysis.

Immunohistochemical staining
Immunostaining was conducted using the Vectastain ABC kit (Vector) in accordance with the manufacturer’s instructions.
Briefly, the slides were deparaffinized, rehydrated, and treated with a citric acid solution to prepare them for immunohistochemical studies. After blocking endogenous peroxidase activity by preincubation in 3% hydrogen peroxide solution, the slides were incubated in blocking solution (PBS, 3% bovine serum albumin) and then sequentially incubated with primary antibodies. The sections were counterstained with hematoxylin (Sigma) for nuclear staining. Negative control slides without primary antibodies did not exhibit nonspecific staining. The slides were independently evaluated by two investigators who were blinded to the experimental data.

Statistical analysis
Data were expressed as the mean±SD. At least three independent experiments were performed. Differences in measured variables between the experimental and control groups were assessed using Student’s t-test. P values less than 0.05 were considered statistically significant. All analyses were performed using GraphPad Prism 5.0.

Results
Paclitaxel or cisplatin induces CTSL protein expression in A549 cells
To determine the effect of chemotherapeutics on the expression level of CTSL, we treated A549 cells with a gradient concentration of paclitaxel or cisplatin at 12, 24, and 48 h. Western blotting was performed to determine the expression of CTSL. As shown in Figure 1A, CTSL clearly increased after treatment of the human cancer A549 cell line with 10 ng/mL paclitaxel for 48 h. Similarly, CTSL markedly increased after the treatment of A549 cells with 0.2 µg/mL cisplatin for 12 h (Figure 1B). These results suggested that paclitaxel and cisplatin induced CTSL protein expression in A549 cells.

CTSL is overexpressed in cisplatin-resistant and paclitaxel-resistant A549 cells
Drug resistance occurs after long-term chemotherapy. Furthermore, paclitaxel and cisplatin induce CTSL protein expression. Therefore, we need to determine whether CTSL expression may be related to the development of drug resistance. To verify this conjecture, A549/TAX (paclitaxel-resistant A549 cells) and A549/DDP (cisplatin-resistant A549 cells) were introduced to conduct the following experiment. First, MTT analysis was performed to test the IC_{50} and the resistance index (RI) values of the A549/DDP and A549/TAX cells. As shown in Supplementary Figure 1A, the IC_{50} values of A549 and A549/DDP cells to cisplatin were 1.5 and 6.0 µg/mL, respectively, and the RI was 4.0; the IC_{50} values of the A549 and A549/TAX cells to paclitaxel were 0.08 and 16 µg/mL, respectively, and the RI was 4.0; the IC_{50} of A549/DDP or A549/TAX cells to cisplatin or paclitaxel was then determined using the MTT assay. The results showed that the IC_{50} of A549/DDP cells to cisplatin decreased by approximately 2.8-fold to cisplatin (Figures 2B and 2E) and approximately 105-fold to paclitaxel (Figure 2D and 2F). These results confirmed that CTSL played an important role in the regulation of acquired drug resistance in A549 cells.

CTSL regulates cisplatin and paclitaxel resistance in A549 cells
The above studies demonstrated that CTSL was overexpressed in A549/DDP and A549/TAX cells. Thus, we hypothesized that drug resistance was modulated by CTSL in A549 cells. To confirm this hypothesis, we silenced CTSL by transfecting the A549/DDP and A549/TAX cells with CTSL siRNA, or by overexpressing CTSL by infecting A549 cells with lentivirus, and the efficiency was detected by Western blotting and immunofluorescence (Figures 1D–1G). The effect of CTSL knockdown on the IC_{50} of A549/DDP or A549/TAX cells to cisplatin or paclitaxel was then determined using the MTT assay. The results showed that the IC_{50} of A549/DDP cells to cisplatin decreased by approximately 1.4-fold (Figure 2A) and that of A549/TAX cells to paclitaxel decreased by approximately 80-fold (Figure 2C). The MTT and colony formation assay results suggested that the forced expression of CTSL increased the IC_{50} of A549 cells by approximately 2.8-fold to cisplatin (Figures 2B and 2E) and approximately 105-fold to paclitaxel (Figure 2D and 2F). These results confirmed that CTSL played an important role in the regulation of acquired drug resistance in A549 cells.

Cisplatin or paclitaxel-resistant A549 cells exhibit an EMT-like phenotypic change
The acquisition of paclitaxel resistance is associated with a more aggressive and invasive phenotype in prostate cancer[28]. It illustrates a possible relationship between drug resistance and invasive potential, as further confirmed by our results. Accumulating evidence has highlighted EMT as the mechanism by which differentiated epithelial cells undergo remarkable morphological changes and acquire more motile and invasive capabilities[29–31]. Here, we found that A549/DDP and A549/TAX cells were spindle-shaped and exhibited reduced cell contact (Figure 3A). The cytoskeletal reorganization is also a characteristic of EMT, and we observed this feature by F-actin staining. In A549/TAX and A549/DDP cells, increased lamellipodia and stress fibers were observed (Figure 3B). To further verify the occurrence of EMT, the migration and invasion ability of cells were evaluated by wound-healing and Transwell assays. The results indicated that cell migration and invasion were dramatically increased in A549/DDP and A549/TAX cells compared with A549 cells (Figure 3C, 3D). EMT is characterized by the combined loss of epithelial markers (E-cadherin and cytokeratin) and the induction of mesenchymal markers (N-cadherin and vimentin)[32]. Particularly, E-cadherin is a key component of adherens junction complexes that maintain cell-cell adhesion and cytoskeletal organization[34–36].

Using Western blotting and immunofluorescence, we found that compared with A549 cells, the expression of E-cadherin decreased and the expression of N-cadherin increased both in A549/TAX and in A549/DDP cells (Figure 3E, 3F). In contrast, cytokeratin 18 decreased, and vimentin expression increased in A549/TAX; however, no obvious changes were
observed in A549/DDP cells. These results showed that EMT was induced in A549/TAX and A549/DDP cells, especially in A549/TAX cells.

**CTSL plays a vital role in mediating EMT in A549/DDP and A549/TAX cells**

EMT has been recognized as a key element for cell migration, invasion, and drug resistance in several types of cancer. Blocking and reversing EMT may be important strategies for overcoming chemotherapy resistance. Based on the above experimental results, we hypothesized that the regulation of CTSL to drug resistance is achieved by mediating EMT.

To confirm the role of CTSL in the regulation of EMT, we suppressed CTSL by transfecting siRNA into A549/DDP and A549/TAX cells. As shown in Figure 4D, the stress fibers in A549/DDP siControl and A549/TAX siControl cells appeared as lamellipodia and extensive parallel bundles, which were densely stained and exhibited well-organized structures. In contrast, the lamellipodia disappeared, and the parallel bundles were disrupted in siCTSL cells, which also demonstrated

---

**Figure 1.** Paclitaxel or cisplatin induces CTSL protein (26 kDa) expression in A549 cells. (A) A549 cells were treated with different concentrations of PTX for 12, 24, and 48 h. Western blotting was performed to detect the expression of CTSL protein. (B) A549 cells were treated with different concentrations of DDP for 12, 24, and 48 h, and the expression level of CTSL was determined by Western blotting. (C) Western blot analysis was performed to determine the expression levels of CTSL in A549, A549/DDP, and A549/TAX cells. (D and F) Western blot and immunofluorescence analyses were performed to determine the expression level of CTSL in A549/DDP and A549/TAX cells transfected with CTSL siRNAs targeting the human CTSL sequence or the control siRNA. (E and G) Western blot and immunofluorescence analyses were performed to determine the expression level of CTSL in A549 cells transfected with LV-Over-CTSL, which targets the human CTSL sequence or the LV-Vector. At least three independent experiments were performed.
Figure 2. CTSL regulates cisplatin and paclitaxel resistance in A549 cells. (A) Effect of CTSL inhibition on the sensitivity of A549/DDP to cisplatin. (C) Effect of CTSL inhibition on the sensitivity of A549/TAX to paclitaxel. (B and D) Effect of CTSL overexpression on the sensitivity of A549 cells to cisplatin. (E and F) Effect of CTSL overexpression on the sensitivity of A549 cells to paclitaxel. Mean±SD. n=3. *P<0.05, **P<0.01 compared with the control. ***P<0.01 compared with the A549-Vector group.
Figure 3. EMT was induced in A549/DDP and A549/TAX cells. (A) Cell morphology was observed by microscopy. Images were captured using a 10x objective lens. (B) Representative phalloidin staining to observe the actin cytoskeleton in A549, A549/DDP, and A549/TAX cells. Cell spreading was analyzed after plating on collagen-coated slides. At the indicated time points, the cells were fixed, stained with phalloidin, and visualized by fluorescence microscopy. Images were captured using a 63x objective lens. (C) The cell migration ability was evaluated by the wound-healing assay. Images were captured using a 4x objective lens. (D) The cell invasion ability was evaluated using a Transwell assay. Images were captured using a 10x objective lens. (E) E-cadherin, N-cadherin, cytokeratin-18, and vimentin were assessed by Western blot analysis. (F) E-cadherin, N-cadherin, and vimentin were subjected to immunofluorescence microscopy. Images were captured using a 63x objective lens. Mean±SD. n=3. *P<0.05, **P<0.01 compared with the control.
Figure 4. CTSL inhibition regulates cisplatin or paclitaxel resistance by reversing EMT in A549/DDP and A549/TAX cells. (A) Effect of CTSL inhibition on cell migration. Images were captured using a 4× objective lens. (B) Effect of CTSL inhibition on cell invasion. Images were captured using a 10× objective lens. (C) Effect of CTSL inhibition on E-cadherin, N-cadherin, cytokeratin-18, and Vimentin were measured by Western blotting. (D) Effect of CTSL inhibition on the actin cytoskeleton, E-cadherin, N-cadherin, and vimentin. F-actin, E-cadherin, N-cadherin, and vimentin were subjected to immunofluorescence microscopy. Images were captured using a 63× objective lens. Mean±SD. n=3. *P<0.05, **P<0.01 compared with the control.
loosely organized F-actin. Moreover, suppression of CTSL attenuated the invasion and migration ability of A549/DDP and A549/TAX cells (Figures 4A and 4B). In addition, CTSL knockdown in A549/DDP and A549/TAX cells led to a significant decrease in N-cadherin and vimentin, along with an increase in E-cadherin and cytokeratin-18 (Figures 4C and 4D).

Furthermore, CTSL overexpression induced morphologic changes (Figure 5A) and an increase in lamellipodia, focal adhesion, and stress fibers in A549 cells (Figure 5E). Wound-healing and Transwell assays were performed to evaluate the effect of CTSL overexpression on cell motor ability. The results suggested that CTSL overexpression increased the invasion and migration ability of A549 cells (Figure 5B and 5C). The Western blotting and immunofluorescence results showed that forced CTSL expression resulted in a decrease in E-cadherin and cytokeratin-18 but induced an increase in N-cadherin and vimentin expression in A549 cells (Figure 5D and 5E). These results support the hypothesis that CTSL regulates cisplatin and paclitaxel resistance by blocking EMT.

CTSL modulates the transcription and expression of EMT-associated transcription factors

We have confirmed that CTSL enhances drug resistance by promoting EMT, but the underlying mechanism remains unclear. Aberrant expression of EMT transcription factors contributes to the appearance of an invasive phenotype by suppressing E-cadherin and inducing EMT in a wide variety of human cancers. In this study, we evaluated whether CTSL upregulation mediated EMT by regulating EMT transcription factors. The expression of E-cadherin is controlled by several transcriptional regulators, including Twist, Snail, Snail2/Slug, E47, ZEB1/TCF8, and ZEB2/SIP1, which bind to E-boxes in the E-cadherin promoter. Western blot and q-PCR analyses were performed to assess the expression of Snail, Slug, ZEB1, and ZEB2. The Western blot results revealed an increase in Snail expression in A549/TAX and A549/DDP cells and of Slug expression in A549/PTX cells; however, there were no obvious changes in A549/DDP cells (Figure 6A). Next, we performed immunofluorescence assays to study the localization of CTSL and Snail in A549 cells, and the results suggested that CTSL and Snail co-localized in A549 cells. Additionally, the q-PCR results suggested that ZEB1 and ZEB2 increased in A549/TAX and A549/DDP cells (Figure 6E). These results indicated that EMT might be mediated by transcription factors.

To further confirm the above results, we knocked down CTSL by siRNA in A549/TAX and A549/DDP cells and overexpressed CTSL in A549 cells. The Western blot and q-PCR results demonstrated that CTSL knockdown reduced the expression of Snail, ZEB1, and ZEB2 in A549/TAX and A549/DDP cells. The level of Slug also decreased in A549/TAX cells (Figure 6B and 6F). Furthermore, CTSL overexpression promoted the expression of Snail, Slug, ZEB1, and ZEB2 in A549 cells (Figures 6C and 6G). These results indicated that CTSL regulated lung cancer cell EMT by modulating EMT-associated transcription factors.

CTSL overexpression attenuates the sensitivity of A549 cells to paclitaxel in vivo

The in vitro results showed that the effect of CTSL on drug resistance was more significant in A549/TAX than in A549/DDP cells. Thus, to further validate the role of CTSL in the regulation of drug resistance, two clones of A549 derivative cells overexpressing CTSL (A549-Over-CTSL) and the control (A549-Vector) were injected subcutaneously into athymic nude mice to generate the mouse model.

After intraperitoneal injection of paclitaxel five times, the tumors were removed from the mice. As shown in Figure 7A, a remarkable decrease in tumor size was observed in the paclitaxel-treated groups compared with the control group in A549-Vector tumors, especially in the high-concentration group. However, a weak decrease in tumor size was determined in the paclitaxel-treated groups compared with the control group in A549-Over-CTSL tumors. The relative growth rate was found to be much higher in A549-Over-CTSL than in A549-Vector tumors (Figure 7A and 7C). These results indicated that overexpression of CTSL enhanced the tolerance of A549 cells to paclitaxel in vivo.

In addition, compared with the control groups (A549-Vector and A549-Over-CTSL), the expression of CTSL protein apparently increased in the PTX-treatment groups (A549-Vector+PTX and A549-Over-CTSL+PTX) (Figure 7D), which is consistent with the experimental results in vitro (Figure 1). The Western blotting results showed that activation of CTSL, either by inducing paclitaxel treatment or by overexpressing CTSL through A549-Over-CTSL, significantly induced EMT compared with the cells without paclitaxel or LV-Vector. As shown in Figure 7E and 7F, the expression of the epithelial markers E-cadherin and cytokeratin-18 decreased, whereas the expression of the mesenchymal markers N-cadherin and vimentin increased following treatment with paclitaxel or overexpressed CTSL. Paclitaxel or CTSL induced the expression of Snail, Slug, ZEB1, and ZEB2 proteins in the control without paclitaxel or A549-Vector (Figure 7E and 7F). These results confirmed that CTSL enhanced drug resistance by promoting EMT via EMT-associated transcription factors in vivo.

Discussion

Lung cancer remains the leading cause of cancer mortality worldwide. To date, chemotherapy remains the mainstream treatment for this disease. However, the administration of chemotherapy is usually limited by the failure of therapy because of resistance. Acquired chemoresistance is a major cause of clinical treatment failure and cancer mortality, and it is often accompanied by an increase in cellular growth pathways and enhanced metastatic potential. Therefore, investigation of the molecular mechanism conferring chemotherapy resistance is urgently needed.

EMT is a key step in the progression of tumors toward metastasis and invasion. EMT has been established as a mechanism that confers tumor cells with abilities that are essential for drug resistance, metastasis, and acquired tumor stem cell traits. Specific targeting of EMT can potentially
Figure 5A–5D. CTSL overexpression induces EMT in A549 cells. (A) CTSL overexpression induces morphological changes in A549 cells. (B) Effect of CTSL overexpression on cell migration. Images were captured using a 4x objective lens. (C) Effect of CTSL overexpression on cell invasion. Images were captured using a 10× objective lens. (D) Effects of CTSL overexpression on E-cadherin, N-cadherin, cytokeratin-18, and vimentin measured by Western blotting. Mean±SD. n=3. *P<0.05, **P<0.01 compared with the control.
decrease metastasis and overcome drug resistance. Peinado et al demonstrated that the development of paclitaxel resistance in EOC cells is accompanied by a transition from an epithelial to a mesenchymal phenotype. Jia et al found that Slug-mediated EMT promotes cell motility and contributes to the acquisition of anoikis resistance. These studies have demonstrated that blocking or reversing EMT is a critical strategy for overcoming chemoresistance. Thus, finding a target to block or reverse EMT is important. In recent years, the molecular mechanisms and signaling pathways mediating EMT were studied intensely, leading to the discovery of several pathways that are involved in the loss of epithelial cell polarity and the acquisition of mesenchymal phenotypic traits.

CTSL is a lysosomal enzyme that is thought to play a key role in malignant transformation. CTSL has been confirmed to be upregulated in a variety of malignancies, including breast, lung, gastric, and colon carcinomas, as well as melanomas and gliomas. In recent years, Zheng et al proposed that CTSL is involved in the regulation of drug resistance. The results revealed that CTSL-induced nuclear accumulation of doxorubicin in drug-resistant cells occurred in a P-gp-independent manner and that CTSL inhibition stabilized and enhanced the availability of cytoplasmic and nuclear protein drug targets, including estrogen receptor-α, Bcr-Abl, topoisomerase-IIa, histone deacetylase 1, and androgen receptor. Therefore, we suppose that CTSL may be a vital target to prevent the development of drug resistance, and the underlying mechanisms may involve multiple signaling pathways. To our knowledge, only a few studies have linked CTSL and EMT with drug resistance. Thus, we explored the relationship between CTSL and EMT in the regulation of resistance to cisplatin and paclitaxel in A549 cells.

First, we found that paclitaxel or cisplatin treatment of A549 cells induced the expression of CTSL protein, which is overexpressed in cisplatin-resistant and paclitaxel-resistant cells compared with parental cells. Subsequent silencing of CTSL reversed the drug resistance in A549/DDP and A549/TAX cells, and overexpression of CTSL attenuated the sensitivity of A549 cells to cisplatin and paclitaxel. These results suggest that CTSL is involved in mediating cisplatin or paclitaxel resistance in A549 cells.

We also found that EMT was not only a cause of cisplatin or paclitaxel resistance in A549 cells but also potentially a consequence of cisplatin or paclitaxel resistance in A549 cells, resulting in increased cancer metastasis after long-term treatment with cisplatin or paclitaxel. We found that compared with A549 cells, A549/DDP and A549/TAX cells underwent morphological and cytoskeletal changes, and their cell invasion and migration abilities increased. The progression of carcinoma cells to metastatic tumor cells frequently involves EMT-like epithelial plasticity changes towards a migratory, fibroblastoid phenotype, which is particularly evident at the invasive front of human tumors. Therefore, we evaluated the expression of EMT marker proteins by Western blot. The results showed that the expression of epithelial markers (E-cadherin and cytokeratin-18) decreased while that of mesenchymal markers (N-cadherin and vimentin) increased in A549/DDP and A549/TAX cells. These results indicated that EMT was induced in cisplatin-resistant and paclitaxel-resistant cells. We also found that CTSL knockdown reversed EMT in A549/DDP and A549/TAX cells. In contrast, overexpression of CTSL induced EMT in A549 cells. Based on the aforementioned results, we propose that the regulation of CTSL during cisplatin and paclitaxel resistance may be achieved by mediat-
Figure 6. CTSL modulates the transcription and expression of EMT-associated transcription factors. (A) The levels of Snail and Slug in A549/DDP and A549/TAX cells were detected by Western blot analysis. (B) Effect of siCTSL transfection on Snail and Slug. (C) Effect of overexpressed CTSL on Snail and Slug. (D) Immunofluorescence was performed to examine the co-localization of CTSL and Snail in A549 cells. (E) The levels of ZEB1 and ZEB2 in A549/DDP and A549/TAX cells were analyzed by q-PCR, and GAPDH served as an internal normalization control. (F) Effect of siCTSL transfection on ZEB1 and ZEB2. (G) Effect of CTSL overexpression on ZEB1 and ZEB2. Mean±SD. n=3. *P<0.05, **P<0.01 compared with the control.
Figure 7A–7E. CTSL overexpression attenuates the sensitivity of A549 cells to paclitaxel in vivo. Vector control or CTSL-overexpressing A549 cells were injected into nude mice, which were treated with the indicated concentrations of paclitaxel five times after two weeks. (A) The tumor size was measured using a Vernier caliper. (B) Body weight was determined using an electronic balance. (C) At the end of the treatment, the tumors were removed from the nude mice. (D, E) Tumor lysates were resolved by SDS-polyacrylamide gel electrophoresis and Western blot analysis using anti-CTSL, anti-E-cadherin, anti-N-cadherin, anti-cytokeratin-18, or anti-vimentin antibodies. β-Actin was used as a loading control. Mean±SD. n=3. *P<0.05, **P<0.01 compared with the control.
ing EMT in A549 cells.

Cytokeratin-18, vimentin, and Slug were upregulated in A549/TAX cells, whereas no obvious changes were detected in A549/DDP cells. Therefore, there may be differences between A549/DDP cells and A549/TAX cells with respect to the mechanism of drug resistance; however, this hypothesis requires further validation.

We have revealed that CTSL overexpression is responsible
for the EMT phenomenon, and these events are associated with cisplatin and paclitaxel resistance in A549/DDP and A549/TAX cells, but the underlying mechanisms remain largely unclear. The hallmark of EMT in cancer is the downregulation of E-cadherin, which is also thought to be a repressor of invasion and metastasis. E-cadherin is a key component of the adherent junction complexes that maintain cell-cell adhesion and cytoskeletal organization. Gocheva et al identified E-cadherin as a target substrate of CTSL, which can be degraded by CTSL. Moreover, E-cadherin downregulation is carried out by the nuclear factors Snail, Slug, ZEB1, and ZEB2, which bind directly to its gene promoter. In the present study, we found that CTSL upregulation was accompanied by increased transcription and expression of EMT-associated nuclear factors. Based on the above results, we concluded that CTSL upregulation is responsible for EMT by upregulating the expression of Snail, Slug, ZEB1, and ZEB2 (Figure 8).

We also found that Snail, a key transcriptional repressor of E-cadherin expression in EMT, exhibited the most remarkable increase in A549/TAX and A549/DDP. Snail is regulated by various signals from the tumor microenvironment. CUX1 is a member of the homeodomain family of DNA-binding proteins and is processed proteolytically to smaller active isoforms by CTSL, such as p110, p90, and p75. p110 CUX1 binds to the Snail and Slug gene promoters; it also activates their expression and then cooperates with these transcription factors to repress the E-cadherin gene. Moreover, NF-κB can bind to the human Snail promoter in the region between -194 and -78 bp and increase the transcription of Snail. Our group found that CTSL acts as an upstream regulator of NF-κB activation. Thus, we suspected that the regulation of CTSL by Snail may be achieved via NF-κB or CUX1. The mechanism by which cisplatin or paclitaxel resistance induced CTSL overexpression in A549 cells and the signaling pathway mediating CTSL upregulation-induced EMT in A549/TAX and A549/DDP cells should also be elucidated in future studies.

We demonstrated that the regulation of chemoresistance by CTSL is mediated through its effect on EMT because the functional status of CTSL affects the expression of EMT-associated transcription factors. This finding may expand current knowledge of CTSL and provide new insights into the mechanism by which drug resistance is regulated in tumor cells. Thus, CTSL may represent a novel therapeutic target to prevent tumor cells from becoming resistant to chemotherapy and reinforce the efficiency of paclitaxel and cisplatin against lung cancer.

Abbreviations
CTSL, cathepsin L; EMT, epithelial-mesenchymal transition; PTX, paclitaxel; DDP, cisplatin; PI3K, phosphatidyl inositol 3-kinase; FGF-2, fibroblast growth factor-2; RI, resistance index; TGF-β, transforming growth factor-β; NF-κB, nuclear factor-κB; ZEB1, zinc finger E-box binding homeobox 1; ZEB2, zinc finger E-box binding homeobox 2; CUX1, cut-like homeobox 1; MTT, methyl thiazolyl tetrazolium.

Acknowledgements
This work was supported by grants from the National Natural Science Foundation of China (Grant No. 30873052, 81072656, 81102466, and 81373430) and the Outstanding Medical Academic Leader Program of Jiangsu Province (Grant No. L201139).

Author contribution
Zhong-qin LIANG and Mei-ling HAN participated in research design; Mei-ling HAN, Yi-fan ZHAO, Cai-hong TAN, Long WANG, and Wen-juan WANG conducted experiments; Mei-ling HAN, Ya-jie XIONG, Yao FEI, and Feng WU contributed to the manuscript preparation. Figure 8. Influence of CTSL on drug resistance in A549 cells.

Influence of CTSL on drug resistance in A549 cells.
new reagents or analytic tools; Mei-ling HAN performed data analysis; and Mei-ling HAN wrote the manuscript.

Supplementary information
Supplementary information is available on the website of Acta Pharmacologica Sinica.

References
1. Kallianos A, Tsimploukis S, Zarogoulidis P, Darwiche K, Charpdou A, Tsioulis I, et al. Measurement of exhaled alveolar nitrogen oxide in patients with lung cancer: a friend from the past still precious today. Onco Targets Ther 2013; 6: 609–13.
2. Maslyar DJ, Jahan TM, Jablons DM. Mechanisms of and potential treatment strategies for metastatic disease in non-small cell lung cancer. Semin Thorac Cardiovasc Surg 2004; 16: 40–50.
3. Smith W, Khuri FR. The care of the lung cancer patient in the 21st century: a new age. Semin Oncol 2004; 31: 11–5.
4. Liu J, Meisner D, Kwong E, Wu XY, Johnston MR. Translymphatic delivery of new reagents or analytic tools; Mei-ling HAN performed data analysis; and Mei-ling HAN wrote the manuscript.

Supplementary information
Supplementary information is available on the website of Acta Pharmacologica Sinica.

References
1. Kallianos A, Tsimploukis S, Zarogoulidis P, Darwiche K, Charpdou A, Tsioulis I, et al. Measurement of exhaled alveolar nitrogen oxide in patients with lung cancer: a friend from the past still precious today. Onco Targets Ther 2013; 6: 609–13.
2. Maslyar DJ, Jahan TM, Jablons DM. Mechanisms of and potential treatment strategies for metastatic disease in non-small cell lung cancer. Semin Thorac Cardiovasc Surg 2004; 16: 40–50.
3. Smith W, Khuri FR. The care of the lung cancer patient in the 21st century: a new age. Semin Oncol 2004; 31: 11–5.
4. Liu J, Meisner D, Kwong E, Wu XY, Johnston MR. Translymphatic delivery of new reagents or analytic tools; Mei-ling HAN performed data analysis; and Mei-ling HAN wrote the manuscript.

Supplementary information
Supplementary information is available on the website of Acta Pharmacologica Sinica.

References
1. Kallianos A, Tsimploukis S, Zarogoulidis P, Darwiche K, Charpdou A, Tsioulis I, et al. Measurement of exhaled alveolar nitrogen oxide in patients with lung cancer: a friend from the past still precious today. Onco Targets Ther 2013; 6: 609–13.
2. Maslyar DJ, Jahan TM, Jablons DM. Mechanisms of and potential treatment strategies for metastatic disease in non-small cell lung cancer. Semin Thorac Cardiovasc Surg 2004; 16: 40–50.
3. Smith W, Khuri FR. The care of the lung cancer patient in the 21st century: a new age. Semin Oncol 2004; 31: 11–5.
4. Liu J, Meisner D, Kwong E, Wu XY, Johnston MR. Translymphatic delivery of new reagents or analytic tools; Mei-ling HAN performed data analysis; and Mei-ling HAN wrote the manuscript.

Supplementary information
Supplementary information is available on the website of Acta Pharmacologica Sinica.

References
1. Kallianos A, Tsimploukis S, Zarogoulidis P, Darwiche K, Charpdou A, Tsioulis I, et al. Measurement of exhaled alveolar nitrogen oxide in patients with lung cancer: a friend from the past still precious today. Onco Targets Ther 2013; 6: 609–13.
2. Maslyar DJ, Jahan TM, Jablons DM. Mechanisms of and potential treatment strategies for metastatic disease in non-small cell lung cancer. Semin Thorac Cardiovasc Surg 2004; 16: 40–50.
3. Smith W, Khuri FR. The care of the lung cancer patient in the 21st century: a new age. Semin Oncol 2004; 31: 11–5.
4. Liu J, Meisner D, Kwong E, Wu XY, Johnston MR. Translymphatic delivery of new reagents or analytic tools; Mei-ling HAN performed data analysis; and Mei-ling HAN wrote the manuscript.

Supplementary information
Supplementary information is available on the website of Acta Pharmacologica Sinica.

References
1. Kallianos A, Tsimploukis S, Zarogoulidis P, Darwiche K, Charpdou A, Tsioulis I, et al. Measurement of exhaled alveolar nitrogen oxide in patients with lung cancer: a friend from the past still precious today. Onco Targets Ther 2013; 6: 609–13.
2. Maslyar DJ, Jahan TM, Jablons DM. Mechanisms of and potential treatment strategies for metastatic disease in non-small cell lung cancer. Semin Thorac Cardiovasc Surg 2004; 16: 40–50.
3. Smith W, Khuri FR. The care of the lung cancer patient in the 21st century: a new age. Semin Oncol 2004; 31: 11–5.
4. Liu J, Meisner D, Kwong E, Wu XY, Johnston MR. Translymphatic delivery of new reagents or analytic tools; Mei-ling HAN performed data analysis; and Mei-ling HAN wrote the manuscript.

Supplementary information
Supplementary information is available on the website of Acta Pharmacologica Sinica.

References
1. Kallianos A, Tsimploukis S, Zarogoulidis P, Darwiche K, Charpdou A, Tsioulis I, et al. Measurement of exhaled alveolar nitrogen oxide in patients with lung cancer: a friend from the past still precious today. Onco Targets Ther 2013; 6: 609–13.
2. Maslyar DJ, Jahan TM, Jablons DM. Mechanisms of and potential treatment strategies for metastatic disease in non-small cell lung cancer. Semin Thorac Cardiovasc Surg 2004; 16: 40–50.
3. Smith W, Khuri FR. The care of the lung cancer patient in the 21st century: a new age. Semin Oncol 2004; 31: 11–5.
4. Liu J, Meisner D, Kwong E, Wu XY, Johnston MR. Translymphatic delivery of new reagents or analytic tools; Mei-ling HAN performed data analysis; and Mei-ling HAN wrote the manuscript.

Supplementary information
Supplementary information is available on the website of Acta Pharmacologica Sinica.

References
1. Kallianos A, Tsimploukis S, Zarogoulidis P, Darwiche K, Charpdou A, Tsioulis I, et al. Measurement of exhaled alveolar nitrogen oxide in patients with lung cancer: a friend from the past still precious today. Onco Targets Ther 2013; 6: 609–13.
2. Maslyar DJ, Jahan TM, Jablons DM. Mechanisms of and potential treatment strategies for metastatic disease in non-small cell lung cancer. Semin Thorac Cardiovasc Surg 2004; 16: 40–50.
3. Smith W, Khuri FR. The care of the lung cancer patient in the 21st century: a new age. Semin Oncol 2004; 31: 11–5.
4. Liu J, Meisner D, Kwong E, Wu XY, Johnston MR. Translymphatic delivery of new reagents or analytic tools; Mei-ling HAN performed data analysis; and Mei-ling HAN wrote the manuscript.

Supplementary information
Supplementary information is available on the website of Acta Pharmacologica Sinica.

References
1. Kallianos A, Tsimploukis S, Zarogoulidis P, Darwiche K, Charpdou A, Tsioulis I, et al. Measurement of exhaled alveolar nitrogen oxide in patients with lung cancer: a friend from the past still precious today. Onco Targets Ther 2013; 6: 609–13.
2. Maslyar DJ, Jahan TM, Jablons DM. Mechanisms of and potential treatment strategies for metastatic disease in non-small cell lung cancer. Semin Thorac Cardiovasc Surg 2004; 16: 40–50.
3. Smith W, Khuri FR. The care of the lung cancer patient in the 21st century: a new age. Semin Oncol 2004; 31: 11–5.
4. Liu J, Meisner D, Kwong E, Wu XY, Johnston MR. Translymphatic delivery of new reagents or analytic tools; Mei-ling HAN performed data analysis; and Mei-ling HAN wrote the manuscript.

Supplementary information
Supplementary information is available on the website of Acta Pharmacologica Sinica.

References
1. Kallianos A, Tsimploukis S, Zarogoulidis P, Darwiche K, Charpdou A, Tsioulis I, et al. Measurement of exhaled alveolar nitrogen oxide in patients with lung cancer: a friend from the past still precious today. Onco Targets Ther 2013; 6: 609–13.
2. Maslyar DJ, Jahan TM, Jablons DM. Mechanisms of and potential treatment strategies for metastatic disease in non-small cell lung cancer. Semin Thorac Cardiovasc Surg 2004; 16: 40–50.
3. Smith W, Khuri FR. The care of the lung cancer patient in the 21st century: a new age. Semin Oncol 2004; 31: 11–5.
4. Liu J, Meisner D, Kwong E, Wu XY, Johnston MR. Translymphatic delivery of new reagents or analytic tools; Mei-ling HAN performed data analysis; and Mei-ling HAN wrote the manuscript.

Supplementary information
Supplementary information is available on the website of Acta Pharmacologica Sinica.

References
1. Kallianos A, Tsimploukis S, Zarogoulidis P, Darwiche K, Charpdou A, Tsioulis I, et al. Measurement of exhaled alveolar nitrogen oxide in patients with lung cancer: a friend from the past still precious today. Onco Targets Ther 2013; 6: 609–13.
2. Maslyar DJ, Jahan TM, Jablons DM. Mechanisms of and potential treatment strategies for metastatic disease in non-small cell lung cancer. Semin Thorac Cardiovasc Surg 2004; 16: 40–50.
3. Smith W, Khuri FR. The care of the lung cancer patient in the 21st century: a new age. Semin Oncol 2004; 31: 11–5.
4. Liu J, Meisner D, Kwong E, Wu XY, Johnston MR. Translymphatic delivery of new reagents or analytic tools; Mei-ling HAN performed data analysis; and Mei-ling HAN wrote the manuscript.
by promoting epithelial-mesenchymal transition via snail2. Oncotarget 2015; 6: 8914–28.

38 Byles V, Zhu L, Lovaas JD, Chmielewski LK, Wang J, Faller DV, et al. SIRT1 induces EMT by cooperating with EMT transcription factors and enhances prostate cancer cell migration and metastasis. Oncogene 2012; 31: 4619–29.

39 Pettit J. The cadherin superfamily. WormBook 2005; 1–9.

40 Eger A, Aigner K, Sonderregger S, Dampier B, Oehler S, Schreiber M, et al. Delta EF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. Oncogene 2005; 24: 2375–85.

41 Huber MA, Azoitei N, Baumann B, Grunert S, Sommer A, Pehamberger H, et al. NF-kappaB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. J Clin Invest 2004; 114: 569–81.

42 Thiery JP. Epithelial-mesenchymal transitions in tumor progression. Nat Rev Cancer 2002; 2: 442–54.

43 Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene 2010; 29: 4741–51.

44 Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer 2007; 7: 415–28.

45 Jia J, Zhang W, Liu JY, Chen G, Liu H, Zhong HY, et al. Epithelial mesenchymal transition is required for acquisition of anoikis resistance and metastatic potential in adenoid cystic carcinoma. PLoS One 2012; 7: e51549.

46 Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol 2006; 7: 131–42.

47 Huber MA, Azoitei N, Baumann B, Grunert S, Sommer A, Pehamberger H, et al. NF-kappaB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. J Clin Invest 2004; 114: 569–81.

48 Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 2014; 15: 178–96.

49 Bucci C, Thomsen P, Nicoziani P, McCarthy J, van Deurs B. Rab7: A key to lysosome biogenesis. Mol Biol Cell 2000; 11: 467–80.

50 Huang DS, Duan HY. Cisplatin resistance in gastric cancer cells is associated with HER2 upregulation-induced epithelial mesenchymal transition. Sci Rep 2016; 6: 20502.

51 Yang J, Weinberg RA. Epithelial-mesenchymal transition: At the crossroads of development and tumor metastasis. Dev Cell 2008; 14: 818–29.

52 Savanger P. Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. Bioessays 2001; 23: 912–23.

53 Gocheva V, Zeng W, Ke D, Klimstra D, Reinheckel T, Peters C, et al. Distinct roles for cysteine cathepsin genes in multistage tumorigenesis. Genes Dev 2006; 20: 543–56.

54 Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, et al. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. Nat Cell Biol 2004; 6: 931–40.

55 Hunt EM, Saykally JN, Anose BM, Kalli KR, Sanders MM. Expression of the ZEB1 (deltaEF1) transcription factor in human: additional insights. Mol Cell Biochem 2008; 318: 89–99.

56 De Herreros AG, Peiro S, Nassour M, Savagner P. Snail family regulation and epithelial mesenchymal transitions in breast cancer progression. J Mammary Gland Biol Neoplasia 2010; 15: 135–47.

57 Kedinger V, Nepveu A. The roles of CUX1 homeodomain proteins in the establishment of a transcriptional program required for cell migration and invasion. J Cell Sci 2005; 20: 348–52.

58 Kedinger V, Sansrequet L, Harada R, Vadnais C, Cadieux C, Fathers K, et al. p110 CUX1 homeodomain protein stimulates cell migration and invasion in part through a regulatory cascade culminating in the repression of E-cadherin and occludin. J Biol Chem 2009; 284: 27701–11.

59 Ramdzan ZM, Nepveu A. CUX1, a haploinsufficient tumour suppressor gene overexpressed in advanced cancers. Nat Rev Cancer 2014; 14: 673–82.

60 Barbera MJ, Puig I, Dominguez D, Julien-Grille S, Guaita-Esteruelas S, Peiro S, et al. Regulation of Snail transcription during epithelial to mesenchymal transition of tumor cells. Oncogene 2004; 23: 7345–54.

61 Yang N, Wang P, Wang WJ, Song YZ, Liang ZQ. Inhibition of cathepsin L sensitizes human glioma cells to ionizing radiation in vitro through NF-kB signaling pathway. Acta Pharmacol Sin 2015; 36: 400–10.