Lentivirus-mediated RNA interference of clusterin enhances the chemosensitivity of EJ bladder cancer cells to epirubicin *in vitro*

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Abstract. Clusterin (CLU) is a glycoprotein that is overexpressed in a number of malignant tumors and has been proven to correlate closely with the chemoresistance of several cancer cells to chemotherapeutic agents. However, the effect of CLU expression on the chemoresistance of bladder cancer to epirubicin remains unknown. In the present study, we aimed to elucidate the role of CLU in the chemoresistance of bladder cancer cells to epirubicin. Lentivirus-mediated RNA interference was applied to knock down CLU in EJ bladder cancer cells. The efficiency was examined by RT-PCR and western blot analysis. After stable CLU silencing, an EJ cell line was established and cells were treated with or without epirubicin. Cell viability, migration, invasiveness, clone formation and cell cycle progression were assessed by MTT assay, wound healing assay, Matrigel invasion assay, plate clone formation assay and flow cytometry, respectively. The results indicated that lentivirus-mediated RNA interference effectively silenced CLU at the RNA and protein levels. CLU knockdown increased the cytotoxicity of epirubicin to EJ bladder cancer cells. Combined treatment with lentivirus-mediated shRNA targeting CLU and epirubicin had maximum effects in bladder cancer cells on cell viability, migration, invasiveness and clone-forming ability. Furthermore, cell cycle analysis indicated that CLU knockdown reinforced the efficacy of epirubicin on G0/G1 cell cycle arrest. Taken together, our results suggest that CLU silencing enhances chemosensitivity of EJ bladder cancer cells to epirubicin. Lentivirus-mediated shRNA targeting CLU may be an alternative approach in the treatment of bladder cancer.

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Introduction

Bladder cancer is the second most common type of cancer in the genitourinary tract and the fourth most common cause of cancer in males in Western industrialized countries (1). Bladder cancer leads to approximately 145,000 deaths per year worldwide (2). Approximately 80% of bladder cancers are initially diagnosed as non-muscle-invasive tumors (3). Transurethral resection (TUR) is the recommended treatment for non-muscle-invasive tumors. However, there is a high incidence of recurrence and progression with TUR treatment. To decrease the incidence of recurrence and progression of these tumors, subsequent intravesical instillation of chemotherapeutic agents are regularly enrolled after TUR. Epirubicin is one of the most common drugs used in intravesical instillation. It is important in the treatment of bladder cancer and decreases the incidence of recurrence (4). Despite this decrease, recurrence of this malignant tumor following TUR with subsequent intravesical instillation remains up to 60-70% within two years. A total of 15-25% of non-muscle-invasive bladder cancers may eventually progress to muscle invasive tumors (1). Studies have mainly attributed this progression to the chemotherapeutic resistance of cancer cells to epirubicin (5). Therefore, it is significant to study the mechanism of chemotherapeutic resistance and enhance the chemosensitivity of cancer cells to chemotherapeutic drugs.

Our previous studies (6) determined that an oncogenic protein, clusterin (CLU), was overexpressed in the majority of bladder cancer cells and therefore may serve as a prognostic molecular marker. Follow-up showed that patients with bladder cancer with a high CLU expression had a higher incidence of recurrence than that of the low CLU expression, even with epirubicin treatment (6). This result indicated that the expression of CLU affected the chemosensitivity of bladder cancer cells to epirubicin and indirectly affected the incidence of recurrence and progression of this malignant tumor. Other studies have also demonstrated the potential role of CLU on chemotherapeutic resistance in several cancer cells, including lung cancer, esophageal carcinoma and prostate tumor (5,7-9). However, no studies have yet examined the role of CLU on chemotherapeutic resistance of bladder cancer cells to epirubicin.

In this study, we aimed to determine whether the regulation of CLU expression affected the chemosensitivity of bladder cancer cells to epirubicin. Lentivirus-mediated RNA interference was applied to permanently silence the expression of CLU in the EJ bladder cancer cell line which, for the first time, had a high expression level of CLU. The silencing efficacy of the lentivirus vector in EJ cells at the protein and mRNA levels were examined. Cell viability, migration, invasiveness, clone formation and cell cycle were examined to assess the role of CLU on the chemosensitivity of EJ bladder cancer cells to epirubicin.

Materials and methods

Cell culture and reagents. Human bladder transitional cell lines T24 and 5637 were purchased from the American Type Culture Collection (Rockville, MD, USA). The EJ and BIU-87 cells were kindly supplied by the Institute of Urology, Beijing Medical University (Beijing, China). Cells were maintained in RPMI-1640 (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) at 37˚C in humidified air containing 5% CO_2 in a monolayer as previously described (10). The 293T cell line, which stably expressed the SV40 large T antigen and facilitated the optimal production of viruses, was obtained from Genechem Co., Ltd., (Shanghai, China) and was cultured in Dulbecco's modified Eagle's Medium (DMEM) with 10% FBS. Epirubicin was purchased from Zhejiang Hisun Pharmaceutical Co., Ltd (Zhejiang, China).

Lentivirus construction and infection. PGCSIL-GFP, a third generation self-inactivating lentivirus vector containing a CMV-driven GFP reporter and a U6 promoter upstream of cloning restriction sites was used in this shRNA silencing system. The synthetic oligonucleotide primers used were: CLU; forward (5'-ccggaaccagagctcgcccttctacttcaagaga gtagaagggcgagctctggttttttg-3') and reverse (5'-aattcaaaaaaa ccagagctcgcccttctactctcttgaagtagaagggcgagctctggtt-3'). The primers were annealed and linked into the cloning restriction site of the vector which had been digested with the restriction enzymes *Age*I and *Eco*RI. The constructed vector PGCSIL-GFP-CLU is able to produce a shRNA targeting 5'-ccaga gctcgcccttctac-3', which is located on the nucleotide position 740-758 of the CLU mRNA (NM_203339). It has been proven to be efficient in CLU silencing experiments (11). The negative control sequence (5'-ttctccgaac gtgtcacgt-3') was used as previously described (12). The NC-shRNA was designed as follows: forward, 5'-ccggaattctccgaacgtgtcacgtttcaagagaac gtgacacgttcggagaatttttttg-3' and reverse, 5'-aattcaaaaaaattct ccgaacgtgtcacgttctcttgaaacgtgacacgttcggagaatt-3'. Following PCR and sequencing of the constructed vector, it was co-transfected with pHelper 1.0 and pHelper 2.0 into 293T cells to package and produce the shRNA expressing lentivirus. The supernatant was collected and concentrated 48 h after co-transfection. The titer of lentivirus targeting CLU (LV-CLU) and lentivirus targeting negative control (LV-NC) was examined by the hole-by-dilution titer method. The vectors and oligonuleotide primers were purchased from Genechem.

To knock down the CLU in the EJ bladder cancer cell line, cells were seeded in a 6-well tissue culture plate with $2x10^5$/well 1 day prior to infection. The complete culture solution was replaced by infection enhancing solution with 5 μg /ml polybrene (Genechem) and the packed lentivirus was added to the cells with multiplicity of infection (MOI) 20. Twelve hours later, the lentivirus solution was replaced with complete culture solution. Infected cells were subcultured every 5-7 days.

RT-PCR. To examine the effect of lentivirus-mediated shRNA at the mRNA level, total RNA was extracted from cell lines by TRIzol/chloroform extraction (Invitrogen Life Technologies, Carlsbad, CA, USA). The purity and concentration of RNA was determined using a spectrophotometer. A total of 1 μg RNA was used for reverse transcription and the Primescript™ reverse transcriptase (Takara Bio, Inc., Shiga, Japan) with random 6-mer primers was used for cDNA synthesis according to the manufacturer's instructions. A total of 1 μl of each first-strand reaction was subsequently used in the PCR amplifications. Oligonucleotide primers used in the PCR were: CLU; 5'-cgcaaggcgaagaccagta-3' and 5'-gaccctcc aagcgatcagc-3'; GAPDH; 5'-gttcgacagtcagccgcatct-3' and 5'-cctgcaaatgagccccagcct-3'. PCR conditions were as follows: denaturation for 5 min at 94˚C, then 30 sec at 94˚C, annealing for 30 sec at 58˚C, 31 sec at 72˚C for 32 cycles and extenstion for 7 min at 72˚C. The products of the PCR amplifications were separated by 1% agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide.

Western blot analysis. For western blot analysis, cells were seeded in 6-well plates at $2x10^5$/well. The cells were grown to 90-100% confluence, and the cells were then collected with 80 μl/well RIPA containing 1 mM phenylmethanesulfonyl fluoride (PMSF). The protein concentration was measured using a BCA protein assay (ThermoScientific Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Total protein (50 μg) was used to run electrophoresis on 10% SDS-polyacrylamide gels. After transferring the protein to polyvinylidene fluoride membranes, the membrane was blocked with 5% non-fat milk for 1 h and immunoblotted with primary antibody at 4˚C overnight. The monoclonal CLU antibody was purchased from Millipore (Billerica, MA, USA) and diluted to 1:100 in 5% non-fat milk containing 0.05% Tween-20. α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted to 1:1000 and used as the internal control. After washing the membrane three times with Tris-buffered saline Tween-20, it was blotted with a secondary antibody for 2 h at room temperature. An ECL kit (ThermoScientific Pierce) was used to detect the bands. The quantification of protein was performed by band analysis using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Bethesda, MD, USA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay. The cells were prepared at a concentration of $3x10^4$ cells/ml and dispensed into 96-well plates at 100 μl/well, then incubated for 0, 1, 3 and 5 days. The MTT assay was performed by adding 20 μl of MTT (5 mg/ml) and incubating for 4 h. When the MTT incubation was complete, the supernatants were removed. A total of 150 μl dimethyl sulfoxide (DMSO) was added to each well and after 15 min the absorbance value (OD) of each well was measured using a microplate reader set at 490 nm. Experiments were performed in triplicate.

Matrigel invasion assay. Cells were trypsinized, washed and resuspended in RPMI-1640 without FBS. Samples of $5x10^4$ cells were placed in the upper chamber of each Transwell device (Falcon, BD Labware, Bedford, MA, USA) with an 8-μm Matrigel-coated polycarbonate membrane filter inserted in 24-well plates. RPMI-1640 with 10% FBS was placed in the lower chamber. After 24 h of incubation, the non-invading cells were removed by wiping the upper surface of the filter with a cotton swab. The remaining cells were fixed in 100% methanol for 20 min, stained with Giemsa (Sigma), and rinsed with distilled water several times. The degree of invasion was quantified by selecting five different predetermined views (original magnification, x200) and counting the cells on the underside of the filters under a microscope.

Wound healing assay. Cells were seeded at $5x10^5$ cells/well in 6-well plates and cultured for ~24 h until almost confluent. The cell monolayer was manually scratched with a pipette tip, washed twice with PBS and allowed to migrate in RPMI-1640 without FBS for 24 h. Phase contrast micrograph images were captured immediately and 22 h after the wound was made. The relative distance traveled by the leading edge from 0 to 22 h was assessed using Adobe Photoshop CS3 software (Adobe Inc.) (n=6).

Plate clone formation assay. The cells were seeded at $1x10^2$ cells/well in 6-well plates and incubated at 37˚C for 14 days. The cells were then washed twice with PBS and fixed in 100% methanol for 20 min, prior to staining with Giemsa solution for 30 min. The number of colonies containing ≥50 cells were counted under a microscope and calculated using the equation: [Plate clone formation efficiency = (number of colonies/number of cells inoculated) x 100%].

Cell cycle analysis. To study the cell cycle progression following RNA interference, the monoparametric FACS analysis method following PI (propidium iodide) staining for total DNA content was conducted as previously described (13). Briefly, after treatment with epirubicin for 24 h, cells from each experimental group were collected and fixed in 70% ethanol at -20˚C overnight. After washing twice with PBS, cells were incubated in PBS containing 100 μg/ml RNase at 37˚C for 30 min. The cells were then stained with 0.5 mg/ml PI for 30 min in the dark at 37˚C. Cell cycle progression was analyzed using a flow cytometer (Beckman Coulter, Miami, FL, USA). During cell cycle analysis, gating and voltage were carefully set to exclude clumped cells and cell debris. The data were analyzed using CXP Analysis 2.0 (Beckman Coulter).

Statistical analysis. Data were presented as the mean ± standard deviation (SD). The statistical correlations in the data between the groups were analyzed by the Student's t-test. $P<0.05$ (two-sided) was considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS 16.0 software (SPSS Inc.).

Results

RT-PCR and western blot analysis of CLU expression in bladder cancer cell lines. To select a cell line with a high level of CLU expression, we used RT-PCR and western blot analysis to analyze the CLU expression at the mRNA and protein levels in BIU-87, 5637, EJ and T24 bladder cancer cell lines. The results suggest that the EJ cell line had the highest expression level of CLU (Fig. 1A).

Establishment of stable CLU-silenced EJ cell line. After the PGCSIL-GFP-CLU vector was constructed, PCR and sequencing analysis were performed. The results obtained proved satisfactory (data not shown). Results of the titer analysis revealed an expression of $3x10^8$ transducing (T) U/ml of LV-CLU versus $5x10^9$TU/ml of LV-NC (13). The EJ cell line, which had the highest level of CLU expression of the four bladder cancer cell lines, was used to study the role of CLU in chemotherapeutic resistance. Four days after infection, the stably infected EJ cells were observed under a fluorescence microscope. The infection efficiency achieved was >90% (Fig. 1B).

Inhibition of CLU expression using lentivirus-mediated shRNA. To identify the specificity and potency of the lentivirus-mediated shRNA inhibition of the CLU expression, the effect of lentivirus on the CLU mRNA and protein levels was determined using RT-PCR and western blot analysis, respectively. As shown in Fig. 1C, five days after lentivirus infection, lentivirus-CLU reduced the CLU mRNA level by 83.3%, while the CLU mRNA expression was almost unaffected by the lentivirus-NC. Inhibition of the CLU protein expression in EJ cells was also observed 5 days after infection (Fig. 1C).

Inhibition of CLU expression enhances the efficiency of epirubicin on cell viability. To determine whether inhibition of the CLU expression by LV-CLU enhances the cytotoxity induced by epirubicin, a cell viability assay was performed after treatment with epirubicin (50 ng/ml) using an MTT assay. The growth curve demonstrated that the LV-CLU-infected EJ cells had a significantly higher susceptibility to epirubicin. CLU knockdown combined with epirubicin treatment had a maximum cytotoxic effect on EJ cells (Fig. 1D).

Inhibition of CLU expression enhances the efficiency of epirubicin on cell invasiveness and migration. Migration and invasion present two important aspects that lead to the ability of cancer cells to metastasize. In this study, a Matrigel invasion assay was used to determine whether CLU knockdown would affect cell invasion. Cells from each experimental group were seeded in transwell chambers and cultured for 24 h. Our results demonstrated that LV-CLU-infected cells combined with epirubicin treatment had minimum invasiveness (Fig. 2A). Statistical analysis revealed a significant difference between the LV-NC and LV-CLU groups with epirubicin treatment ($P<0.01$).

The role of CLU on cell migration was assessed by a classic wound healing assay. LV-NC- and LV-CLU-infected cells were treated with or without epirubicin (50 ng/ml), respectively, for 22 h. The relative cell migrating distance was calculated using Adobe Photoshop CS3 software (Adobe). The data demonstrated a statistically significant difference between the LV-CLU- and the LV-NC-infected cells. The LV-CLU-infected

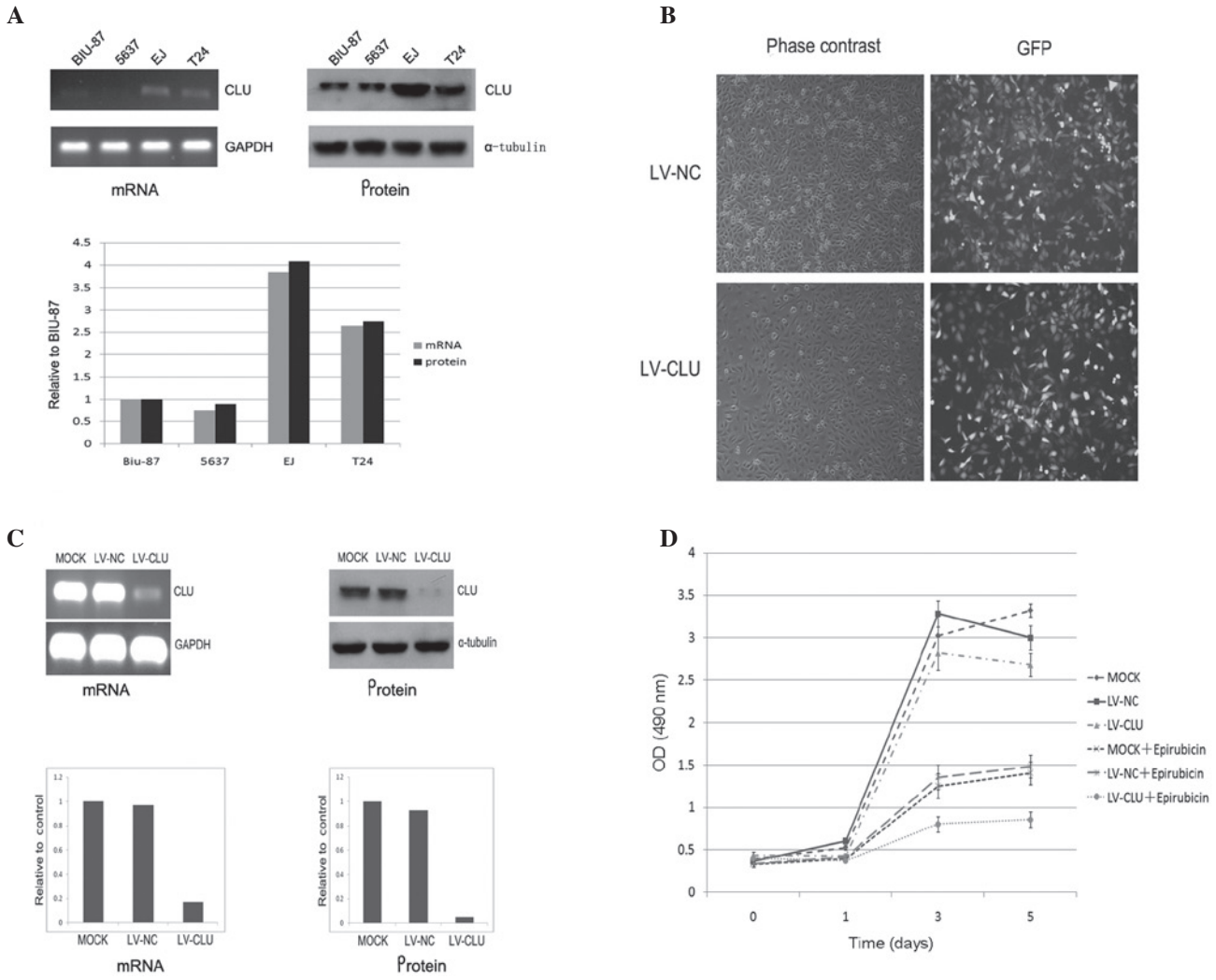

Figure 1. Establishment of stable CLU silencing EJ cell line and detection of the growth velocity. (A) Analysis of levels of CLU expression in mRNA and protein in 5637, BIU-87, EJ and T24 bladder cancer cell lines. Results indicate that the EJ cell line had the highest level of CLU expression. (B) The infection efficiency of EJ cells (MOI 20) is shown. The cells were infected with LV-CLU or LV-NC. Four days later, the infection efficiency achieved was >90% (magnification, x100). (C) Analysis of CLU mRNA expression in the EJ cell line after lentivirus infection. RT-PCR analysis demonstrated that LV-CLU reduced CLU mRNA by 83.3% in EJ cells, while CLU mRNA expression was almost unaffected by the LV-NC. Western blot analysis of the CLU protein expression demonstrated a proximal result. LV-CLU reduced the CLU protein expression by 94.8%. (D) An MTT assay demonstrated the effect of LV-CLU on cell susceptibility to epirubicin. Epirubicin untreated groups have a higher cell viability and proliferation compared to the epirubicin-treated groups. Moreover, in the epirubicin-treated groups, LV-CLU-infected EJ cells had a lower cell viability and proliferation than the mock EJ cells and CLU-NC-infected EJ cells. Therefore, inhibition of the CLU expression increased the cytotoxicity induced by epirubicin (50 ng/ml). CLU, clusterin; LV-CLU lentivirus targeting CLU; LV-NC, lentivirus targeting negative control; MOI, multiplicity of infection.

cells had a lower migration. Thus, CLU silencing enhances the toxicity of epirubicin on cell migration (Fig. 2B).

CLU knockdown increases the toxicity of epirubicin on clone formation. A plate clone formation assay was used to assess the role of CLU on clone formation in bladder cancer cells. Mock EJ cells, LV-NC- and LV-CLU-infected EJ cells were seeded in 6-well plates, respectively, and cultured with or without epirubicin for 14 days. The number of colonies containing ≥50 cells were counted under a microscope. Our results demonstrated that the CLU knockdown EJ cells combined with epirubicin treatment had a clone formation rate of 21±5%, which was lower than that of the other groups (Fig. 2C). Therefore, the CLU knockdown combined with epirubicin treatment effectively decreased the clone formation of EJ bladder cancer cells.

CLU knockdown increases the potency of epirubicin on cell cycle arrest. Following treatment with or without epirubicin (50 ng/ml) in each experimental group, a flow cytometer was used to quantify changes in the cell cycle. In our study, CLU silenced cells demonstrated G0/G1 phase arrest and G2/M and S-phase reduction. A total of 98.3% of cells were blocked in the G0/G1 phase in the CLU knockdown EJ cells, while only 64.9% of cells were blocked in the G0/G1 phase in LV-NC-infected cells after treatment with epirubicin (Fig. 2D). The difference was statistically significant.

Discussion

CLU, also known as SP-40, sulfated glycoprotein 2, testosterone-repressed prostate message-2 and apolipoprotein J was

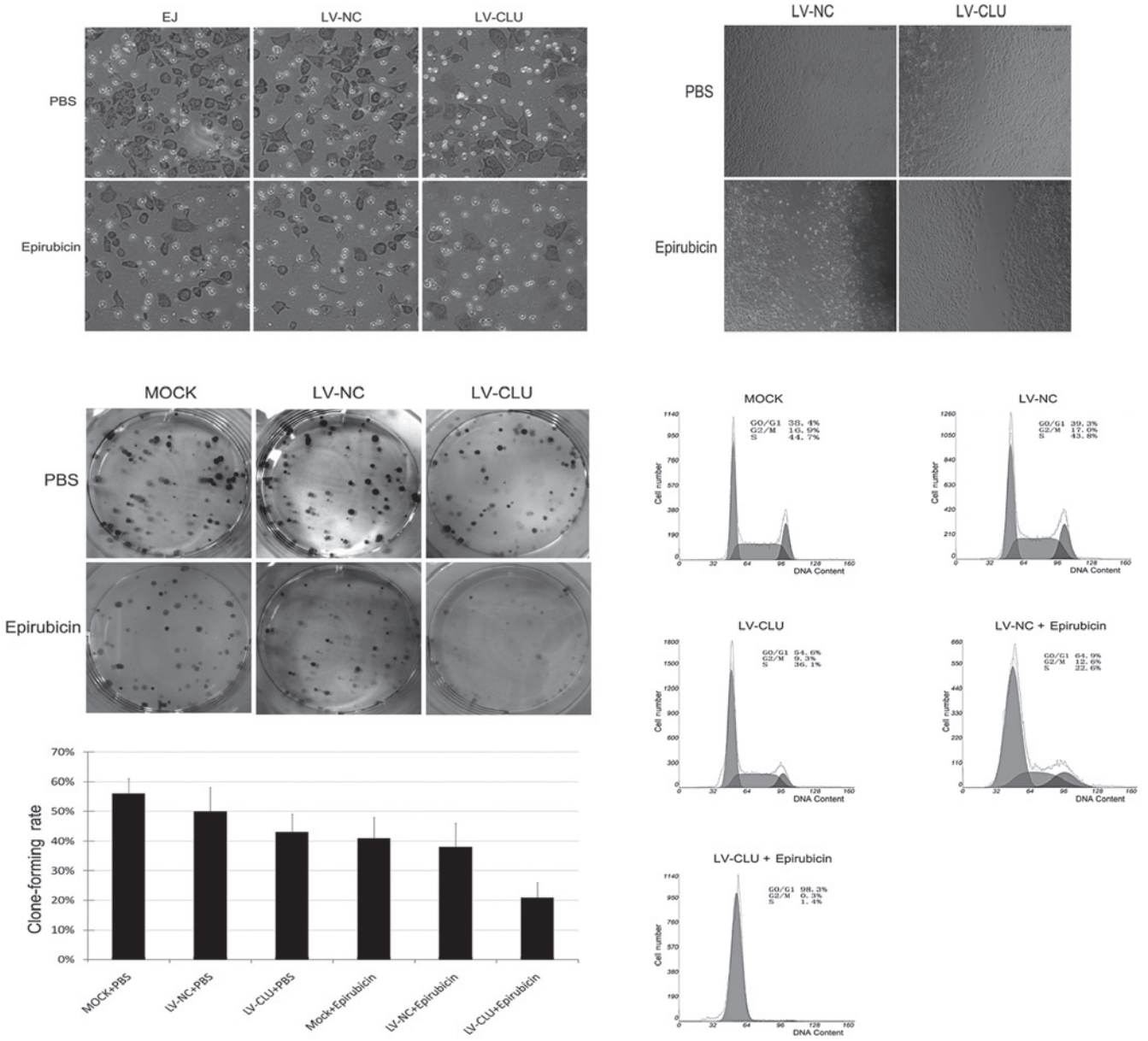

Figure 2. Analysis of the role of CLU in chemotherapeutic resistance. (A) A Matrigel invasive assay demonstrated that LV-CLU-infected cells combined with epirubicin treatment had minimum invasiveness. Statistical analysis revealed a significant difference between the LV-NC and LV-CLU groups with epirubicin treatment ($P<0.01$) (n=5). (B) A wound healing assay was used to detect the migration ability of EJ cells after CLU knockdown. LV-NC- and LV-CLU-infected EJ cells were treated with or without epirubicin, respectively, for 22 h. LV-CLU-infected EJ cells had a greater migrating distance compared to LV-NC infected EJ cells which were almost confluent. A significant difference was observed between the two groups ($P<0.05$) (n=6). (C) A plate clone formation assay with or without epirubicin treatment is shown. The data demonstrated that CLU knockdown EJ cells combined with epirubicin treatment had a lower clone formation rate than the other groups ($P<0.05$) (n=3). (D) Effect of CLU knockdown on the cell cycle detected by flow cytometric analysis. CLU-silenced cells demonstrated G0/G1 phase arrest and G2/M and S-phase reduction. After treatment with epirubicin for 24 h, 98.3% cells were blocked in the G0/G1 phase in CLU knockdown EJ cells, while only 64.9% cells were blocked in the G0/G1 phase in LV-NC-infected cells. The difference was statistically significant. CLU, clusterin; LV-CLU lentivirus targeting CLU; LV-NC, lentivirus targeting negative control.

first isolated from ram rete testis fluid by Blaschuk *et al* (14) in 1983. This single copy gene locates on chromosome 8p12-p21 and encodes an mRNA of approximately 2 kb which produces a glycoprotein of 449 amino acids which is cleaved into an α and β subunit. These appear as a smear of approximately 40 kDa on an SDS polyacrylamide gel (14). CLU is generally expressed in almost all fluids and tissues in humans and is overexpressed in a number of cancers, including breast, colon, bladder and melanoma (15-18). Previous studies have found that the majority of bladder cancers were accompanied with CLU overexpression, and the recurrence-free survival rate of patients with a strong CLU expression was significantly lower than that of patients with a weak expression (16,19). Recent studies have considered CLU as a key contributor to chemoresistance to anticancer agents (5). CLU knockdown was found to significantly chemosensitize cancer cells, including prostate, and breast and lung cancer (9,20-23). However, no study has examined whether CLU silencing is able to chemosensitize bladder cancer cells to

epirubicin, one of the most common drugs, after TUR, used for intravesical instillation in patients with non-muscle-invasive bladder cancer. In this study, we aimed to determine whether CLU knockdown enhances the chemosensitivitiy of bladder cancer cells to epirubicin. Our results demonstrated that a combined treatment of CLU silencing and epirubicin had a maximum cytotoxic effect on bladder cancer cells, with regards to cell proliferation, migration, invasion, clone formation or cell cycle arrest. This finding indicated that silencing of CLU may be an alternative method to treating the chemoresistance of bladder cancer cells to epirubicin.

In this study, lentivirus-mediated shRNA was used, for the first time, to knock down CLU. Despite OGX-11, a second generation of antisense oligonucletide targeting CLU currently being widely used in numerous experiments (24,25), it has certain disadvantages, including transient CLU silencing and low transfection efficiency, particularly in non-dividing primary cells. To overcome these limitations, we designed and constructed lentivirus-mediated shRNA to obtain a stable CLU knockdown effect. Lentivirus-mediated shRNA provides specific, long-lasting silencing and maximal inhibition of gene expression at lower concentrations in a variety of human cells, including primary non-dividing cells and also in the transgenic mouse (26-28). CLU has been proven to be safe for humans and has been used in several clinical trials with no evident side effects (29,30). In our study, we revealed that CLU is a powerful tool which decreased mRNA expression by over 80% and protein expression by over 90%. Thus, we recommend lentivirus-mediated shRNA as an improved method to knock down CLU.

Our study indicates that lentivirus-mediated shRNA targeting CLU effectively sustains the knockdown of CLU gene expression in EJ bladder cancer cells. This study describes the successful construction of a lentivirus RNAi vector targeting CLU that may become a useful tool for the study of the function of the CLU gene in bladder cancer cells. Our findings markedly suggest that CLU is important in the chemoresistance of bladder cancer cells to epirubicin. CLU knockdown combined with epirubicin treatment may prove to be a more effective approach for controlling recurrence and progression than epirubicin treatment alone in intravesical instillation. It represents a novel approach to regulating bladder cancer recurrence and progression.

Acknowledgments

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