Small interfering RNA targeted to secretory clusterin blocks tumor growth, motility, and invasion in breast cancer

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Clusterin/apolipoprotein J (Clu) is a ubiquitously expressed secreted heterodimeric glycoprotein that is implicated in several physiological processes. It has been reported that the elevated level of secreted clusterin (sClu) protein is associated with poor survival in breast cancer patients and can induce metastasis in rodent models. In this study, we investigated the effects of sClu inhibition with small interfering RNAs (siRNAs) on cell motility, invasion, and growth in vitro and in vivo. MDA-MB-231 cells were transfected with pSuper-siRNA/sClu. Cell survival and proliferation were examined by 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and clonogenic survival assay. The results showed that sClu silencing significantly inhibited the proliferation of MDA-MB-231 cells. The invasion and migration ability were also dramatically decreased, which was detected by matrigel assays. TUNEL staining and caspase-3 activity assay demonstrated that sClu silencing also could increase the apoptosis rate of cells, resulting in the inhibition of cell growth. We also determined the effects of sClu silencing on tumor growth and metastatic progression in an orthotopic breast cancer model. The results showed that orthotopic primary tumors derived from MDA-MB-231/pSuper-sClu siRNA cells grew significantly slower than tumors derived from parental MDA-MB-231 or MDA-MB-231/pSuper scramble siRNA cells, and metastasize less to the lungs. These data suggest that secretory clusterin plays a significant role in tumor growth and metastatic progression. Knocking-down sClu gene expression may provide a valuable method for breast cancer therapy.

Keywords clusterin; siRNAs; breast cancer; metastasis; apoptosis; gene treatment

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targeting clusterin in breast cancer

survival. However, whether the inhibition of clusterin leads to cell survival or cell death is not entirely clear.

In breast cancer, three small retrospective studies have shown an association with elevated clusterin expression and large tumor size, estrogen and progesterone receptor status, and with the progression from the primary carcinoma to metastatic carcinoma in lymph nodes in breast cancer [22,28,29]. Nevertheless, suppression of sClu could enhance ionizing radiation lethality and chemosensitize human breast cancer cells [30,31]. In this study, we knocked down sClu in the MDA-MB-231 cells with high expression level of sClu using small interfering RNA (siRNA), and observed that sClu silencing induced growth retardation and suppressed invasion and metastasis in vitro and in vivo.

Materials and Methods

Cell lines and cell culture

MDA-MB-231 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, USA). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Burlington, Canada) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37°C. 293T cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Burlington, Canada) supplemented with 10% fetal calf serum (FCS), and 100 units/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37°C.

sClu siRNA
	sClu siRNAs were purchased from Dharmacon (Lafayette, USA). sClu siRNA sequence 5'-GAGCGCAGACUCUCAUCAU-3' was corresponded to the human clusterin initiation site. A scramble control 5'-CAGCGCUGACACAGUUCAU-3' was used.

Stable cell line selection

sClu silencing cells were selected in MDA-MB-231 cells and MDA-MB-231/pSuper scramble siRNA cells with retrovirus vectors pSuper (Clontech, Mountain View, USA) following the manufacturer’s instructions. Briefly, sClu siRNA was cloned into pSuper vectors, and the recombinant plasmid was cotransfected into 293T cells with plasmid PegPam3 and RDF to produce the virus-containing recombinant plasmid. Viral supernatants were collected and transduced into MDA-MB-231 cells. Stable cell lines were selected by adding 0.5–1 μg/ml of puromycin. At least three individual clones were selected for each stable cell line. The silencing of sClu in each stable cell lines was confirmed by western blot analysis.

Western blot analysis

Briefly, MDA-MB-231/pSuper sClu siRNA, MDA-MB-231/pSuper scramble siRNA, and MDA-MB-231 cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in cold lysis buffer [10 mM Tris HCl, pH 8.0, 240 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mM PMSF, 1% Triton X-100, 1 mM sodium vanadate, and 1 g/ml of leupeptin, pepstatin, aprotinin] by incubation at 4°C for 20 min, followed by centrifuging at 14,000 g for 20 min. Protein concentration of the supernatants was determined using a BCA protein assay kit (Pierce, Rockford, USA). Equal amounts of proteins were loaded on 10% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes, and the blots were subsequently probed with the antibodies: anti-sClu (1:100, Santa Cruz, Santa Cruz, USA) and anti-β-actin (1:500, Santa Cruz). For detection, horseradish peroxidase-conjugated secondary antibodies (1:500, Santa Cruz) were used followed by enhanced chemiluminescence development (Millipore, Billerica, USA). Beta-actin was used as a control to normalization. The optical density was quantified using an AlphaEase FC Version 4 analysis software (Alphalmager HP, Alpha Innotech, San Leandro, USA).

3-(4,5-Dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay

Cells were seeded in 96-well plates (2×10^4 per well) and cultured in DMEM plus 10% FCS at 37°C for 24 h. 3-(4,5-Dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (20 μl) mixed with 100 μl of growth medium was added to each well and incubated at 37°C for 2 h. Absorbance was recorded at 490 nm with an EL-800 universal microplate reader (Bio-Tek Instruments Inc., Winooski, USA).

Clonogenic survival assays

Stable transfected MDA-MB-231 cells (5×10^3) were plated in 1 ml of RPMI 1640 (Invitrogen) containing 7.5% FCS, 7.5% PHA-LCM (Hangzhou, China), and 0.3% agar gel (Bactoagar, Difco, Sigma, St Louis, USA). The cells were cultured in Petri dishes for 14 days in a 5% CO2 incubator at 37°C. Then the agar culture was tipped gently on to microscope slides, air-dried, and stained for 1 h with Luxol-Fast-Blue (Sigma). The stain solution was consisted of 0.1 g of stain powder (Gurr MBS, Searle Diagnostic, High Wycombe, UK) dissolved in 70% ethanol saturated with urea. The slides were washed with tap water for 15 min, followed by staining with hematoxylin for 2 min. Under the microscope, stained cells were readily identified by their bright apple-green cytoplasm.

In vit r o terminal transferase dUTP nick end labeling assay

MDA-MB-231, MDA-MB-231/pSuper sClu siRNA, and MDA-MB-231/pSuper scramble siRNA-transfected cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Burlington, Canada) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37°C.
(1 × 10⁴) were cultured on chamber slides for 24 h. Apoptosis of the cells was evaluated on the basis of the terminal transferase dUTP nick end labeling (TUNEL) assay according to the manufacturer’s instructions. TUNEL–positive cells were colored using diaminobenzidine (DAB) as the chromogen, and counterstained with hematoxylin. The percentage of TUNEL–positive cells was assessed in five randomly selected fields in each section. All assays were performed in quadruplicate.

**Caspase-3 activity assay**

Caspase-3 activity was determined using the caspase-3/CPP32 colorimetric assay kit (Biovision, Milpitas, USA) according to the manufacturer’s protocol. Cells (2 × 10⁶) were pelleted and lysed with RIPA buffer after transient transfection with pSuper sClu siRNA for 48 h. Cell lysates (100 μg protein per sample) were incubated at 37°C in 50 μl of 2× reaction buffer on 96-well microtiter plates. The reaction buffer was supplemented with 10 mM DTT and 4 mM DEVD-pNA substrates in a final volume of 100 μl and incubated at 37°C for 1.5 h. P-nitroanilide formation was measured using the enzyme-linked immunosorbent assay micro-plate reader at a wavelength of 405 nm. Caspase-3 activities were expressed as percentage of enzyme activity compared with control (untreated cells).

**Motility and invasion assays**

The motile and invasive abilities of the cell lines were measured using a Boyden chamber in a 24-well plate assay system (8 μm pores, Corning Costar, New York, USA). Chemotactic-induced motility in response to an FCS concentration gradient was measured by adding 400 μl of RPMI1640 completed culture medium containing 10% FCS to the lower compartment, and 2 × 10⁵ cells in 200 μl of RPMI1640 completed culture medium, but with 2% FCS to the upper compartment of the Boyden chamber. The cells were incubated for 24 h, the upper side of the filter was wiped with a cotton swab to remove any non-motile cells, and the motile cells on the lower side of the filter were fixed and stained using the Dif-Quik histochemical stain (Dade Behring, Düdingen, Switzerland) according to the manufacturer’s instructions. The lower compartment of the Boyden chamber was checked and the number of stained cells on the lower side of the filter was counted using a Dynascope with a ×20 objective (Vision Engineering, Surrey, UK). For each cell line, four experiments were carried out, each experiment consisting of three filters and 10 fields per filter were counted.

For cell invasion, the filters were coated with 50 μg Matrigel (Becton Dickinson, Oxford, UK) and the experiments were carried out as for the chemotactic motility assays. The number of invasive cells/field on the lower side of the filters was determined as for the motility assays.

**Tumorigenicity studies and metastasis assays**

The MDA-MB-231/pSuper sClu siRNA, MDA-MB-231/pSuper scramble siRNA, and MDA-MB-231 cells (1 × 10⁶ cells in 0.1 ml PBS) were injected into three groups of 6-week-old female nude mice, at a single subcutaneous (s.c.) site in the left inguinal mammary fat pad. For the s.c. tumor model, tumor size was measured weekly using a digital caliper (VWR International, Radnor, USA) and tumor volume was determined with the following formula: tumor volume (mm³) = [length (mm)] × [width (mm)]² × 0.52. Mice were autopsied when the tumors reached ~10% of their body weight after 3 weeks. The tumors, mammary glands, lymph nodes, lungs, and any suspicious looking tissues were fixed in Methacarn, processed and embedded in paraffin wax as for the rat. Samples were sectioned and stained with hematoxylin and eosin. Sections of both lungs and lymph nodes for each animal were examined for metastases.

**Immunohistochemistry**

Tumor samples of the orthotopic xenograft fixed in 10% neutral-buffered formalin were embedded in paraffin using automatic embedding equipment, after which 5 μm sections were prepared. The sections were incubated with the primary antibody anti-sClu (1:100, Santa Cruz) and anti-ki-67 (1:400, Santa Cruz) for 12 h at room temperature. For each case, a corresponding section was incubated in Tris-buffered saline without the primary antibody as a control for non-specific staining. Biotinylated rabbit anti-mouse secondary antibodies (DAKO, Hamburg, Germany) were added for 40 min at room temperature, followed by the avidin-biotinylated peroxidase complex for an additional 40 min. After washing with distilled water for 10 min, staining was achieved using DAB. Then the sections were counterstained with Mayer’s hemalum and mounted for microscopy. All sections were examined by three independent investigators.

**In vivo TUNEL detection**

*In vivo* apoptosis was evaluated by TUNEL staining using the Apoptag Peroxidase *In Situ* Detection Kit (Chemicon, Rosemont, USA) according to the manufacturer’s instructions. Briefly, histological sections were deparaffinized, hydrated in deionized water, and then rinsed with PBS. The sections were treated with 20 μg/ml of proteinase K for 15 min to digest protein, and with 3% H₂O₂ for 5 min to quench endogenous peroxidase activity. After washing with PBS, the equilibration buffer was added. The slides were then treated with 8 μl working strength TdT enzyme at 37°C for 60 min. Subsequently, the sections were incubated with pre-heated working strength TdT enzyme solution for 10 min, with anti-digoxigenin-POD for 30 min, and with Pierce Metal Enhanced DAB for 3–6 min, and washed with PBS after incubation. Finally, the sections were counterstained with Mayer’s hematoxylin and then mounted. Control slides...
were ordered from Serologicals Corporation (Norcross, USA). The results were observed with an optical microscope. The percentage of apoptotic cells was calculated as the number of apoptotic cells per number of total cells \( \times 100\% \).

**Statistical analyses**
Statistical comparisons were performed using Student’s unpaired t-tests (for two groups) or one-way non-parametric analysis of variance for more than two groups. Data are expressed as the mean ± SD or mean ± SEM. \( P < 0.05 \) was considered as significant difference.

**Results**

**sClu silencing decreased cell proliferation**
We detected the expression of sClu protein in the MDA-MB-231/pSuper sClu siRNA, MDA-MB-231/pSuper scramble siRNA, and MDA-MB-231 cells. The results showed that sClu was significantly silenced in MDA-MB-231/pSuper sClu siRNA cells, and pSuper scramble siRNA had no effect on the expression of sClu [Fig. 1(A)]. We further explored the effect of sClu silencing on the cell proliferation. MTS results showed that the proliferation fold of MDA-MB-231/pSuper sClu siRNA cells decreased ~50% compared with MDA-MB-231/pSuper scramble siRNA, and the clone formation ability was also decreased 60% [Fig. 1(B and C)].

**sClu silencing increased cell apoptosis**
TUNEL assay showed that silencing of sClu induced significant apoptosis cells in MDA-MB-231 cells [Fig. 2(A)]. To ascertain whether the activation of caspase-3 played a central role in mediating apoptotic responses, we measured the intracellular levels of caspase-3 in transiently transfected MDA-MB-231 cells/pSuper sClu siRNA at 48 h after transfection. As shown in Fig. 2(B), MDA-MB-231/pSuper sClu siRNA cells exhibited a dramatic increment in caspase-3 activity compared with untreated or MDA-MB-231/pSuper scramble siRNA cells, which indicated that sClu silencing could activate caspase-3 and increase the cell apoptosis.

**sClu silencing inhibited cell motility and cell invasion in vitro**
We cultured the cells using a Boyden chamber in a 24-well plate assay system for 24 h, which could ensure that all motile cells remained attached to the lower side of the filter. Only 32 migrating cells were detected in MDA-MB-231/pSuper sClu siRNA cells per field, which was less than the MDA-MB-231/pSuper scramble siRNA cells (128 migrating cells per field) and controls (130 migrating cells per field), respectively [Fig. 3(A and B)]. To compare cell invasion and cell motility, the invasive abilities of the cell lines were measured using the same assay conditions as the motility assay, except for Matrigel coating on the filter to separate the upper and lower compartment of the Boyden chamber. The invasive ability was significantly lower in the MDA-MB-231/pSuper sClu siRNA cells than in the MDA-MB-231 cell lines and the MDA-MB-231/pSuper scramble siRNA cells (\( P < 0.05 \)) [Fig. 3(C and D)]. A close association was also observed between sClu levels and invasive ability of the MDA-MB-231 cell lines [Fig. 3(C and D)].

**sClu silencing on tumor metastasis and growth in vivo**
One million cells from the MDA-MB-231, MDA-MB-231/pSuper sClu siRNA, and MDA-MB-231/pSuper scramble siRNA cells were injected into the mammary fat pads of female nude mice. The incidence of mammary gland tumors and lung metastases in nude mice injected with cell lines is shown in Table 1. All mice produced mammary gland tumors after 3 weeks (100% incidence), but the metastatic ability was different among the three group cells. Both the MDA-MB-231 and MDA-MB-231/pSuper scramble siRNA cells showed a statistically significant greater

![Figure 1](image-url) **Effects of sClu silencing in MDA-MB-231 cells on cell proliferation, clone formation**  (A) sClu expression in stable cells by western blot analysis. (B) Cells were seeded in 96-well plates \( (2 \times 10^3 \text{ per well}) \) and serum starved. Cell growth was assessed with the MTS assay on 24 h. (C) Cell proliferation was assessed by clone formation assay. Control, MDA-MB-231 cells; sClu siRNA, MDA-MB-231/pSuper sClu siRNA cells; Mock siRNA, MDA-MB-231/pSuper scramble siRNA cells. **\( P < 0.01 \) vs. Control.
number of lung metastases than the MDA-MB-231/pSuper sClu siRNA cells (Table 1, P < 0.005). Metastases were also observed in the inguinal lymph nodes of some of the mice of MDA-MB-231 and MDA-MB-231/pSuper scramble siRNA cells (data not shown).

**sClu siRNA inhibited tumor growth and increased the apoptosis in vivo**

We first detected the expression level of sClu in the orthotopic breast tumors. Immunocytochemical staining showed the higher expression of sClu in MDA-MB-231/pSuper scramble siRNA tumors compared with that in MDA-MB-231/pSuper sClu siRNA tumors [Fig. 4(A)]. We next evaluated the effects of sClu silencing on the growth of tumors in vivo. Figure 4(B) shows that sClu silencing significantly reduced the tumor volume by 3 weeks after initiation of injection (P ≤ 0.05). Furthermore, we investigated the effect of sClu silencing on tumor cell proliferation. Tumor sections from nude mice were assessed for Ki-67 expression, which is an indicator of DNA synthesis. After 3 weeks of treatment, very low numbers of Ki-67\(^+\) tumor cells were observed in pSuper sClu siRNA mice and many Ki-67\(^+\) tumor cells were seen in pSuper scramble siRNA mice [P < 0.05; Fig. 4(C)].

To investigate whether sClu silencing induced apoptosis in MDA-MB-231 tumors xenografts, the TUNEL assay was performed. The tumor cells in pSuper sClu siRNA mice showed typical apoptotic cell morphology with nuclear chromatin condensation and fragmentation, and in pSuper scramble siRNA mice almost no apoptotic tumor cells were detected [P < 0.05; Fig. 4(D)].

**Discussion**

sClu’s function is considered enigmatic. It performs various cellular functions, including cell survival, apoptosis, and tumor progression [32]. Modulation of the migratory-invasive cell behavior represents another function in which the role of sClu is ambiguous.

It has been reported that overexpression of clusterin prolongs cell survival under unfavorable conditions in the metastatic process, resulting in the enhanced metastatic
potential of renal cell carcinoma [33]. In breast cancer in vivo and in vitro, over-expression of sClu promoted invasion and metastasis of orthotopic primary MCF-7 tumors [28]. Whether sClu silencing could inhibit invasion and metastasis of MCF-7 tumors is not completely clear.

Recently, a new method for investigating human gene functions using siRNAs has become available. siRNAs are powerful reagents for post-transcriptional silencing, where mRNA targeted by the siRNAs is degraded in vivo and the level of the encoded protein is reduced. Yang et al. [34] has found that siRNA-mediated IAP genes silencing inhibited invasion of bladder cancer T24 cells. Guo et al. [35] and Meng et al. [36] have shown that siRNA targeting some target genes also suppresses cell proliferation and invasion. Here, we demonstrated that clusterin down-expression decreased invasion and metastatic progression, at least to the lungs, which suggested that the secreted glycoprotein has additional extracellular functions. This kind of decrease in the number of metastatic sites does appear to correlate with the decreased size of the MDA-MB-231/sClu siRNA primary tumors. Knockdown of sClu expression in ovarian cancer cells has similar effects [37]. However, sClu does not affect motility or invasiveness of renal cell carcinoma cells, although it enhances their metastatic potential [33]. Moreover, through stabilizing inhibitors of NF-kB and thereby interfering with NF-κB signalling, sClu inhibits neuroblastoma cell invasion [38]. These apparent discrepancies may be reflecting the specific contribution of different cellular contexts, or alternatively, may have arisen from the existence of different Clu isoforms. An example supporting this notion is the observation that both sClu and nClu inhibit motility of prostate epithelial cells while only nClu affects motility of prostate cancer cells [39].

sClu is an extracellular chaperone that has been functionally implicated in DNA repair, cell-cycle regulation, apoptotic cell death, and tumorigenesis [40]. Clusterin, as an anti-apoptotic mediator [41], over-expression helps confer a chemoresistant phenotype through inhibition of apoptosis in human RCC cells [42]. In breast cancer, over-expression of sClu also blocks the TNFalpha-mediated induction of p21 and abrogates the cleavage of Bax to t-Bax, rendering the MCF-7-CLU cells significantly more resistant to the cytokine than the parental cells. Orthotopic primary tumors derived from MCF-7-CLU cells grow significantly more rapidly than tumors derived from parental MCF-7 cells.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total number of mice</th>
<th>Number of mice with mammary gland tumors (%)</th>
<th>Number of mice with metastases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>18</td>
<td>18 (100%)</td>
<td>12 (66.7%)</td>
</tr>
<tr>
<td>sClu siRNA</td>
<td>20</td>
<td>20 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Mock siRNA</td>
<td>20</td>
<td>19 (95%)</td>
<td>10 (52.6%)</td>
</tr>
</tbody>
</table>

Table 1 Incidence of mammary gland tumors and lung metastases in nude mice injected with cells

*aTotal number of mice injected.

*bNumber of mice with palpable mammary gland tumors 3 weeks after inoculation with cells; percentage of tumor-bearing mice (%) is also shown.

*Number of mice with metastases in the lungs; percentage of tumor-bearing mice with metastases (%) is also shown.

*dSignificantly higher number of metastases than the sClu siRNA cells (P < 0.005).

Figure 4 Effects of sClu silencing on the growth of Orthotopic xenograft

(A) Immunocytochemical staining for sClu in tumor xenografts (x100). (B) Nude mice were inoculated with MDA-MB-231/pSuper sClu siRNA or MDA-MB-231/pSuper scramble siRNA cells and allowed to grow for 3 weeks. The tumor volume was measured. Results are expressed as mean ± SD. n = 20. (C) The intensity of immunohistochemical staining of Ki-67 in tumor xenografts. (D) The intensity of immunohistochemical staining of TUNEL in tumor xenografts (x100). *P < 0.05 vs. Mock siRNA.
[28]. In the distinct cellular contexts of the osteosarcoma and prostate cancer cells assayed, sClu is a central molecule in cell homeostasis that exerts a cytoprotective function. sClu knockdown in human cancer cells induces significant reduction of cellular growth and higher rates of spontaneous endogenous apoptosis. Moreover, sClu knockdown cancer cells were significantly sensitized to both genotoxic and oxidative stress induced by chemotherapeutic drugs and H2O2, respectively [43].

Our results clearly demonstrate that the secretedCLU protein form is an essential molecule for the cellular homeostasis of the MDA-MB-231 cells assayed. In the present study, sClu knockdown resulted in significant growth retardation and higher rates of endogenous apoptosis in vitro and in vivo. The proposed essential role of sClu for cellular homeostasis is clearly supported by the fact that sClu is associated with cells surviving programmed cell death during development [44].

These data demonstrate that sClu, which is frequently up-regulated in breast cancers, may play a significant role in tumor growth and metastatic progression by blocking the apoptotic signaling, leading to cell survival and by increasing the ability of cells to survive during one or more phases of the metastatic process. The sClu siRNA used in this study are potent tools for modulating the Clu gene expression, and they may ultimately be developed into attractive antitumor therapeutics.

References


