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*Original Article*

# SLUG promotes invasion and metastasis of anaplastic thyroid cancer cells through repression of E-cadherin

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**Abstract:** Objective: Overexpression of SLUG has been implicated in promoting migration and invasion of many cancer cells, including anaplastic thyroid cancer (ATC) cells. Thus, targeted down-regulation of SLUG expression in ATC cells and determination of its implications would provide new treatment approaches for disease management. Methods: Small interfering RNA (siRNA) targeting SLUG (SLUG siRNA) was used to develop clonal derivatives of the ATC SW1736 cells. SLUG cDNA transfection was used to restore the SLUG expression in the SLUG siRNA transfected SW1736 cells. E-cadherin siRNA was used to inhibit E-cadherin expression in the SLUG siRNA transfected SW1736 cells. The effect of SLUG and E-cadherin expression on migration and invasion of SW1736 cells in vitro was detected. Further, the effect of targeting SLUG on lung metastasis in an mouse SW1736 cell tumor model was detected. Results: Targeting SLUG expression by SLUG siRNA transfection in SW1736 cells showed a 75% decrease in migration and a 85% decrease in invasion in vitro. Targeting E-cadherin by E-cadherin siRNA transfection or SLUG overexpression by SLUG cDNA transfection restored the invasion and migration ability of SW1736 cells. In vivo analysis indicated a 80% decrease in the number of mice bearing macroscopic lung metastases. Analysis of SLUG signaling pathways in the clonal derivatives showed a decrease in SLUG expression and increase in E-cadherin expression in the SLUG siRNA transfected tumor. Conclusions: Therapies targeting SLUG signaling pathway may be more effective in preventing organ metastasis.

**Keywords:** Anaplastic thyroid cancer, SLUG, metastasis

## Introduction

Anaplastic thyroid cancer (ATC) represents 1%-2% of all thyroid tumors and is characterized by aggressive, local invasion and common distant metastases. Histologically, ATC is characterized by elevated mitotic rate and lymphovascular invasion [1]. One important factor that contributes to the invasiveness of ATC is the epithelial to mesenchymal transition (EMT) of the ATC cells [2, 3]. EMT is a complex cellular process reflecting a high level of phenotypic plasticity, which is marked by the down-regulation of epithelial markers (E-cadherin) and transcriptional induction of mesenchymal markers (vimentin and N-cadherin) [4]. The transition of epithelial cells to mesenchymal cells induces the loss of cell-cell adhesion, cell polarity, and the acquisition of migratory and invasive properties [5]. Understanding the mechanisms that drive EMT is therefore important to identify new targets for the prevention of metastasis in ATC.

SLUG (SNAI2) is a member of the SNAIL family of zinc finger transcriptional repressors that mediates sequence-specific interactions with DNA. The most highly studied members of this family include SNAIL (SNAI1) and SLUG, both of which are conserved among vertebrate species [7]. SNAIL family members have been implicated in a variety of developmental and cellular processes, many of which relate to cell motility and induction of the EMT; Additionally, SNAIL family members are aberrantly expressed in a variety of cancers where they regulate a diverse number of processes ranging from tumor cell

invasion and metastasis to cell survival and proliferation [8].

It has recently found that SLUG was not expressed in cells derived from normal thyroid tissue, or in normal human thyroid samples, but were highly expressed in cell lines derived from thyroid carcinomas, in human thyroid carcinoma samples, and their metastases [9]. Buehler et al. has found that SLUG was negative in follicular adenomas, less in papillary and follicular thyroid carcinomas, but positive in the anaplastic thyroid carcinoma or cell line, suggesting the role of epithelial-mesenchymal transition in the development of anaplastic thyroid carcinoma [10]. In our previous study, we described an important role for SLUG in the invasion and metastasis of extrahepatichilarcholangiocarcinoma [11], esophageal adenocarcinoma [12], bladder cancer [13] and pancreatic cancer [14]. However, it remains to be addressed whether and how SLUG affected the cell invasion and migration of ATC cells.

Now, we have investigated the effect of SLUG inhibition by siRNA or overexpression by SLUG cDNA transfection on invasion and migration on the ATC cells in vitro and vivo. Our results indicated Slug promotes *in vitro* invasion by downregulation of E-Cadherin. Targeting SLUG inhibits *in vitro and vivo* invasion and metastasis by upregulation of E-cadherin expression. We therefore concluded SLUG could be a target for the treatment of ATC.

## Materials and methods

### *Ethics*

All animal studies were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the affiliated hospital of Qingdao University.

### *Cell line and culture*

The human anaplastic thyroid cancer (ATC) SW1736 cell line was purchased from DSMZ (Beijing, China). The cells were cultured as the DSMZ's instruction. Briefly, the cells were grown in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin, sodium pyruvate, and non-essential amino acids. Adherent monolayer cultures were maintained on plastic and incubated at 37°C in 5% carbon dioxide and 95% air. The cultures were free of *Mycoplasma* species. In all of the assays, a monolayer of cells that was 50-70% confluency was used. All the methods used were according to the manufacture's instruction.

### *Plasmid cDNA and siRNA transfection*

Slug siRNA and Slug cDNA was from our laboratory. SW1736 cells growing in 6-well plates were incubated with human Slug siRNAs (100 nmol/L). Mock-transfection was performed using a negative control siRNA (Santa Cruz Biotechnology) as control. Cells were harvested at 48 h post transfection, washed and stored for future experiments. The knockdown efficiency was assessed using the western blot assay. Stably expressed clones, used for in vivo study and invasion study, were selected by using medium containing G418 (600 ug/ml) for 14 days. Cells were routinely maintained in selection media containing 300 ug/ml of G418-sulfate to avoid overgrowth of nontransfected cells. To determine the effect of SLUG overexpression on SW1736 cells invasion, the stably transfected SLUG siRNA/SW1736 cells was transfected with SLUG cDNA (50 nmol/L) for 48 hs used the methods above. To determine the effect of E-cadherin on SW1736 cells invasion, the stably transfected SLUG siRNA/SW1736 cells was transfected with E-cadherin siRNA (100 nmol/L) for 48 hs used the methods above.

### *Western blot analysis*

Cell were trypsinized and washed with cold PBS. Cell pellets were lysed with lysis buffer and incubated on ice for 30 minutes with gentle shake or with ABC protein extraction solution according to the instructions. Protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membrane. Protein blots were probed with an appropriate primary antibody (SLUG and E-cadherin) and a secondary antibody (IRDye, LI-COR) and then analyzed by quantitative immunoblot using an Odyssey Infrared Imaging System (LI-COR). β-actin was used as loading control.

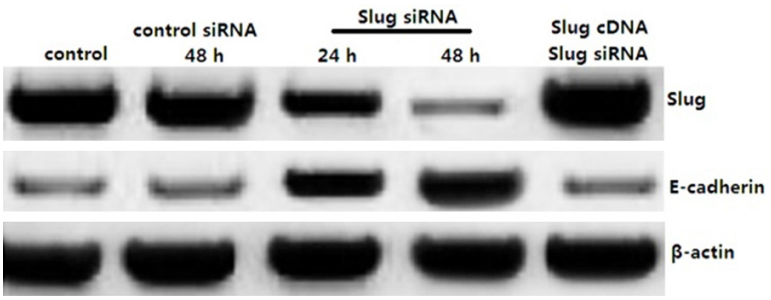


**Figure 1.** Effect of SLUG gene knockdown or overexpression on SLUG and E-cadherin in SW1736 cells.

*Transwell migration and matrigel invasion assays*

Transwell migration and matrigel invasion assays were performed using a transwell membrane (8-μm pore size, 6.5-mm diameter; Corning Incorporated, Corning, NY, USA) in a 24-well plate according to the manufacturer's instructions. A matrigel matrix (1:5 Dilute, 50 μl/well, BD Biosciences) was coated in the transwell membrane (8-μm pore size, 6.5-mm diameter; Corning Incorporated, Corning, NY, USA) and used for the cell invasion assay. The lower chamber of the transwell plates was filled with 600 μl IMDM medium containing 10% FBS. SW1736 cells were detached from the tissue culture plates and resuspended in IMDM medium containing 1% FBS and then loaded to the upper side of the chamber (200 μl/well). For the invasion assay, SW1736 cells ($1 \times 10^5$ cells/well) was used. The cells were placed in incubators at 37°C for different time periods according to preliminary experiments. The filter inserts were then removed from the wells. Cells on the upper surface of the filter were removed using cotton swabs. Those on the lower surface were fixed with 4% paraformaldehyde in PBS, stained with 0.1% crystal violet and counted. Cells that migrated or invaded were counted in five random fields of each filter under a microscope (IX71, OLYMPUS, JAPAN) at 200 × magnification.

*In vivo xenograft model of ATC metastasis*

Four- to 5-wk-old female athymic nude mice were purchased from Shanghai, China. A total of 3 groups consisting of 10 mice each were included in the study. SLUG siRNA-expressing SW1736 cells were injected into the right back of 4- to 5-wk-old athymic mice. Tumors were allowed to grow for 6 wk. Six weeks later, mice were sacrificed and tissues and lungs were collected to determine the amount of metastases. Incidence of metastasis was determined by the presence of macroscopic lesions on the surface of the lung while sacrificing the mice.

*Immunohistochemistry*

5-um sections of paraffin-embedded tumor tissues using antibodies for SLUG and E-cadherin to determine the expression of these protein in the primary tumor material. Antigen retrieval was achieved by incubating the slides in citrate buffer for 20 min in a steamer and endogenous peroxidase was blocked by incubation with 3% $H_2O_2$ for 20 min at room temperature. To determine the protein expression, stained slides were examined under high power (x40). The antigen-antibody complex was visualized with diaminobenzidinetetrahydrochloride and tissues were counterstained minimally with hematoxylin.

*Statistical analysis*

Student's t test or two way ANOVA was used for the statistical analysis of theresults. The differences were considered to be significant when $P<0.05$.

## Results

*Effect of SLUG siRNA transfection on SLUG expression in SW1736 cells*

As shown in **Figure 1**, baseline of SLUG protein expression has a high levels in the SW1736 cells by western blot assay. SW1736 cells transfected with SLUG siRNA displayed a time dependent reduction in the expression levels of SLUG protein, and at 48 hs transfection, SLUG expression reached the lowest levels. Nonsilencing control did not exhibit any effect on protein levels of SLUG in the SW1736 cells. These data confirmed the suppression effect of siRNA and established the efficiency of siRNA transfection.

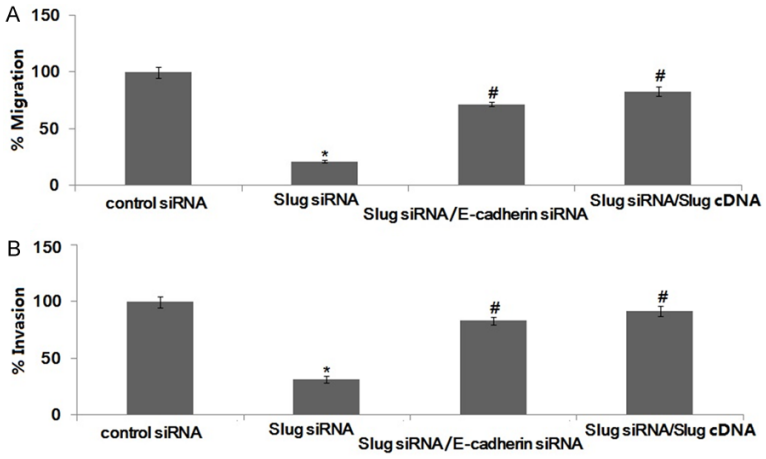


**Figure 2.** SLUG-induced E-cadherin on migration and invasion of ATC cells. SW1736 cells were transfected with SLUG siRNA or/and E-cadherin siRNA or SLUG cDNA, cell migration (A) and invasion (B) was detected bytranswell migration and matrigel invasion assays. Vs control, $^{*}P<0.01$; vs SLUG siRNA, $^{\#}P<0.01$.

*Effect of SLUG cDNA transfection restored the SLUG expression in the SLUG siRNA/SW1736 cells*

The stably SLUG siRNA transfected SW1736 (SLUG siRNA/SW1736) cell were stransfected with SLUG cDNA plasmid, SLUG protein expression was increased compared with the SLUG siRNA/SW1736 cells (**Figure 1**). Because SLUG expression was observed to be the highest levels 36 h after transfection (data no shown), we selected this time point for further studies.

*Effect of SLUG siRNA on invasive capability of SW1736 cells*

The SLUG gene is reported to confer invasive characteristics to various cancer cells; however, its role in the invasion and metastasis of ATC is yet unknown. Next, we analyzed the effect of SLUG gene suppression on the invasive capability of highly invasive and metastatic SW1736 cells by employing an in vitro Transwell migration and matrigel invasion assays.

In transwell migration and matrigel invasion assays, the results showed that the cells in the lower chamber of transwell were obviously decreased in SLUG siRNA transfected SW1736 cells, compared with SW1736 or Nonsilencing siRNA transfected SW1736 cells ($P<0.01$) (**Figure 2A**, **2B**); These results indicate that SLUG knockdown inhibits both the migration and invasion of SW1736 cells.

*Effect of SLUG gene knockdown on E-cadherin expression*

To determine the mechanism through which SLUG controls the invasion and metastasis of SW1736 cells, we detected the E-cadherin expression in both SLUG-knockdown and SLUG overexpressing SW1736 cells by Western blot assay. The results showed that targeting SLUG increased E-cadherin expression in the SW-1736 cells, and SLUG overexpression by SLUG cDNA transfection inhibited E-cadherin expression in the SW1736 cells (**Figure 1**).

*Knockdown of SLUG gene inhibits invasive of SW1736 cells by upregulation of E-cadherin*

Subsequently, we detected the effect of SLUG-induced E-cadherin on cell migration and invasion. The stable SLUG siRNA transfected SW1736 cells was transfected with E-cadherin siRNA for 48 hs, the migratory and invasive ability was restored compared to the SLUG siRNA transfected SW1736 cells (**Figure 2A**, **2B**).

*Analysis of targeting SLUG in a xenograft model of ATC metastasis*

SLUG siRNA/SW1736 cells were implanted in the back of 4- to 5-week-old female athymic nude mice. Cohorts of animals from each group were sacrificed 42 days after removal of the primary tumors to determine lung metastasis. As shown in **Figure 3A**, SLUG expression showed significant decrease in the SLUG siRNA/SW1736 groups than the control groups. However, E-cadherin expression showed significant increase in the SLUG siRNA/SW1736 groups than the control groups. Results shown in **Figure 3B**, a significant decrease in the number of macroscopic metastases present in the SLUG siRNA/SW1736 groups. Although 100% of mice injected with SW1736 cells developed macroscopic lung metastasis, 30% of the mice injected with the SLUG siRNA/SW1736 clones developed similar lung metastasis.

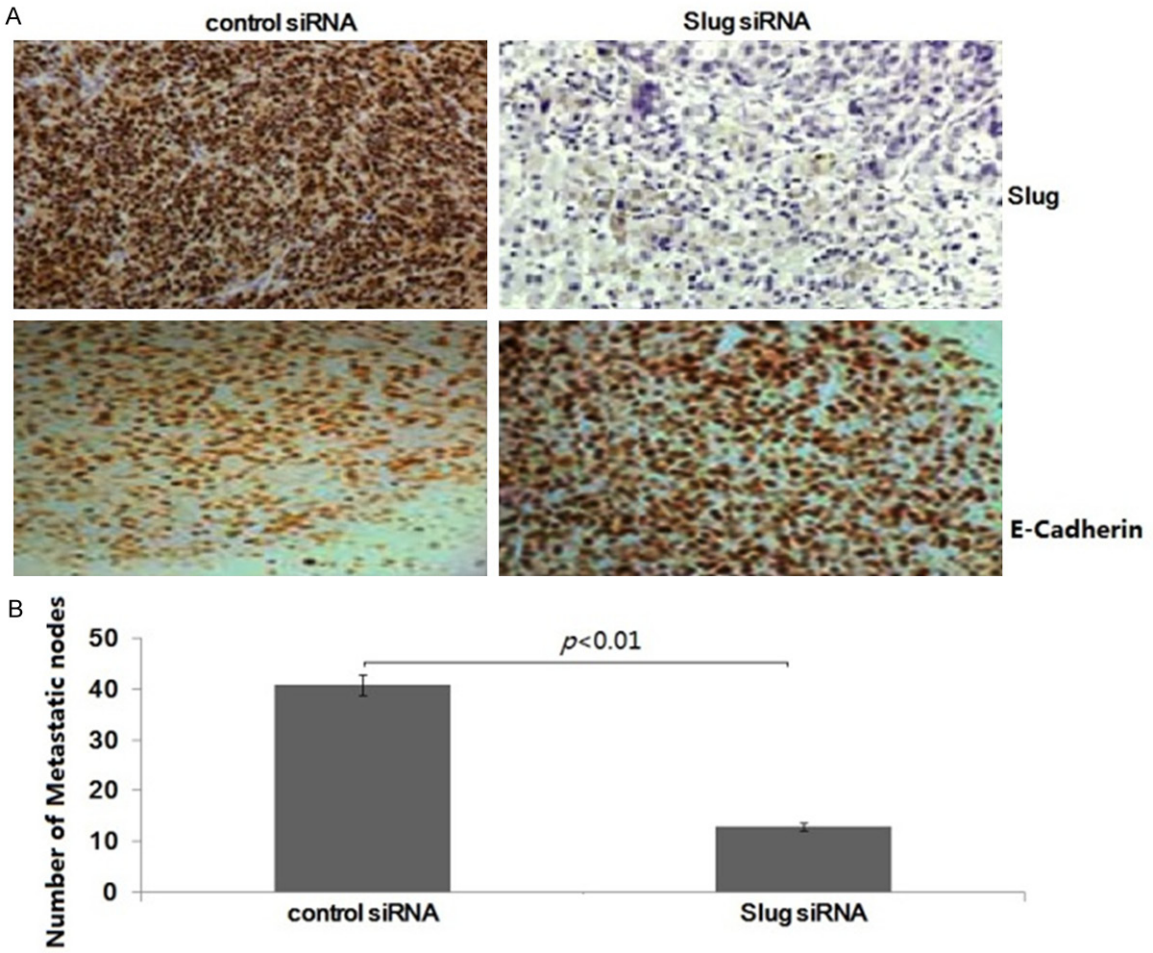


**Figure 3.** Knockdown of SLUG in a xenograft model of ATC metastasis. A. SLUG and E-cadherin was detected by immunohistochemistry. It was showed that SLUG was significantly inhibited by SLUG siRNA, and E-cadherin expression was increased by SLUG siRNA. B. Thenumber of macroscopic metastases in SLUG siRNA groups and control siRNA groups. ($P$<0.01).

## Discussion

SLUG is widely expressed in cancer cells and is thought to promote tumor progression through regulation of cell survival and invasiveness [15-18]. Several studies from our laboratory and others have shown that inhibition of SLUG in both malignant cells in vitro and tumors in vivo reduces cancer cells invasiveness [11-14, 16, 17].

EMT has been considered the critical mechanism involved in cancer metastasis and EMT transcription factors [19], however, the mechanism of EMT transcription factors in human cancers are largely unknown. As strong E-cadherin repressors and major EMT inducers, SLUG play a critical role in the invasion and metastasis of plenty of human cancers [11-18]. However, SLUG promotes invasion and metastasis of ATC cells has not been reported.

Here we found that targeting SLUG abrogates migrative and invasive ability of SW1736 cell in vitro and vivo. Furthermore, restoring the SLUG expression by cDNA transfection could restore the invasive ability of SW1736 cell. In vivo, SLUG knockdown also inhibited the lung metastasis of mice. It was surprising that Slug, alone, would play such a dominant role.

Malignancy of carcinoma cells is characterized by the loss of both cell-cell adhesion and cellular differentiation, and this has repeatedly been reported to correlate with E-cadherin downregulation. Loss of E-cadherin could be attributed to somatic mutations in some tumour types [20], promoter hypermethylation [21] or the

action of transcriptional repressors, such as Slug, Snail, Twist1 and Zeb-1 [22, 23]. Downregulation of E-cadherin is associated with the development of invasive carcinoma, metastatic dissemination, and poor clinical prognosis [24, 25]. Therefore, deregulation of E-cadherin expression may contribute to tumourigenesis.

To determine whether SLUG promotes invasion by repressing the E-cadherin expression, we detected the E-cadherin expression in SLUG siRNA and SLUG cDNA transfected SW1736 cells. The results showed that siRNA-mediated downregulation of SLUG expression increased E-cadherin expression in SW1736 cells, as demonstrated by western blot analysis. However, after the SLUG/siRNA transfected SW1736 cells was transfected with SLUG cDNA or E-cadherin siRNA, the E-cadherin expression was decreased, and cell migratory and invasive ability was restored. Studies in mouse tumour models have also suggested that tumour metastasis can be achieved by SLUG inhibition and E-cadherin upregulation. These data demonstrate that SLUG promotes invasion of SW1736 cells in vitro by repression E-cadherin expression.

## Conclusion

We presented experimental evidence, which strongly supports the pro-metastasis effects of SLUG in ATC cells in vitro and in vivo. Thus, we believe that knockdown of SLUG could potentially be an effective therapeutic method for the activation of E-Cadherin, resulting in the inhibition of cell invasion and metastasis of ATC. Further work is necessary to understand whether the role of other members of SNAIL family in the regulation of E-cadherin expression and cell invasiveness influences metastatic development in ATC cancers.

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## Disclosure of conflict of interest

None.

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