

Lentivirus-delivered Krüppel-like factor 8 small interfering RNA inhibits gastric cancer cell growth *in vitro* and *in vivo*

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Abstract We found that the transcription factor Krüppel-like factor 8 (KLF8) was highly expressed in gastric cancer tissues and cell lines compared with adjacent noncancerous regions and gastric epithelial mucosa cells. We employed a lentivirus-mediated RNAi technique to knockdown KLF8 expression in gastric cancer cell line SGC7901 and observed its effects on cell growth *in vitro* and *in vivo*. Knockdown of KLF8 inhibited SGC7901 cell proliferation, promoted cell apoptosis, inhibited the tumorigenicity of SGC7901 cells, and significantly decreased tumor growth when the cells were injected into nude mice. These results indicated that overexpression of KLF8 may influence the biological behavior of SGC7901 gastric cancer cells. Knockdown of KLF8 expression by lentivirus-delivered siRNA may be useful as a therapeutic agent for the treatment of gastric cancer.

Keywords Gastric cancer · Krüppel-like factor 8 · Proliferation · Apoptosis

Introduction

Gastric cancer is still the second most common cancer in Asia, especially in Eastern Asia, in spite of its incidence gradually declining in western countries [1, 2]. Although the survival of patients with gastric cancer has improved due to advances in diagnostic and therapeutic modalities, overall patient outcome has not improved significantly, and long-term survival remains unsatisfactory due to the high rate of recurrence and metastasis [3, 4]. Therefore, novel strategies to treat gastric cancer are needed.

KLF8 is a member of the Sp/KLF family of transcription factors. Members of this family encode a C-terminal DNA-binding domain with three Krüppel-like zinc fingers [5]. These proteins are thought to play an important role in the regulation of the epithelial-mesenchymal transition (EMT), a process that occurs normally during development but also during metastasis [6]. Recently, studies have also indicated that KLF8 is highly expressed in several types of human cancers [7], including ovarian [8], breast [9], and renal carcinomas [10], and plays an important role in oncogenic transformation and cancer cell invasion. However, the role of KLF8 in gastric cancer is still unknown.

In this study, we confirmed that KLF8 is highly expressed in gastric cancer tissues and cell lines. Subsequently, we employed the newly developed lentivirus-delivered small interfering RNA (siRNA) technique to observe the effect of knockdown of KLF8 on human gastric cancer cell growth *in vitro* and *in vivo*.

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Materials and methods

Tissue samples, cell lines, and cell culture conditions

Tissue samples from 80 primary gastric cancers and their noncancerous counterparts were obtained from patients who underwent surgery at the Department of General Surgery in Xijing Hospital (Xi'an, China). All patients who agreed to have their surgical tissues dissected for this study signed an informed consent. All cases of gastric cancer had been clinically and pathologically proven (data not shown). The protocols used in this study were approved by the hospital's Protection of Human Subjects Committee. The following human gastric adenocarcinoma cell lines were employed: MKN45 and AGS were obtained from the Shanghai Cell Bank (Shanghai, China), and the immortalized gastric epithelial mucosa cell line GES and human gastric adenocarcinoma cell line SGC7901 were from the Academy of Military Medical Science (Beijing, China). Cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum with sodium pyruvate, nonessential amino acids, L-glutamine, a two-fold vitamin solution (Life Technologies, Grand Island, NY, USA), and a penicillin-streptomycin mixture at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Recombinant lentivirus generation

The complementary DNA sequence of KLF8 was designed from the full-length KLF8 sequence by Shanghai GeneChem Company (Shanghai, China). After testing knockdown efficiencies, stem-loop oligonucleotides were synthesized and cloned into the lentivirus-based vector PsicoR (Addgene, Boston, MA, USA). A nontargeting scrambled RNA PsicoR vector was generated as a negative control. Lentivirus particles were prepared as described previously [11]. Gastric cancer cells were infected with the KLF8 siRNA-lentivirus or negative control virus at 7 days and examined at 10 days.

Lentivirus infection

Cells were incubated with lentivirus in a small volume of serum-free DMEM at 37°C for 4 h. Then, 10% DMEM was added, and the cells were placed in an incubator for additional time (as indicated) for the following experiments. The infection efficiency of green fluorescent protein (GFP) in SGC7901 cells was about 90% at a multiplicity of infection (MOI) of 20, and at this concentration, no toxic effect was observed. Thus, the following experiments were performed using viruses at an MOI of 20, unless indicated otherwise. The lentivirus packaged with GFP was used for the cell proliferation assays, and the lentivirus without GFP was used for the cell apoptosis PI staining to exclude from the interference with GFP signal. The SGC7901 cells

transfected with the KLF8-siRNA or scramble siRNA were designated lenti-siRNA/KLF8 and src-siRNA, respectively.

Monolayer growth assay

The monolayer culture growth rate was determined using a Cellomics Arrayscan (Genechem, Shanghai, China). Briefly, after the cells were infected with virus for 3 days, cells of the same density were seeded into flat-bottom 96-well plates and grown under normal conditions. Cultures cells were observed at 0, 1, 2, 3, 4, and 5 days using a Cellomics Arrayscan to observe the cell growth with GFP signal. Subsequently, the growth curve of the cells was measured after each experiment. Each experiment was performed in triplicate.

Colony formation assay

A soft-agar colony formation assay was performed to assess the anchorage-independent growth ability of cells as a characteristic of *in vitro* tumorigenicity using a Cellomics Arrayscan (Genechem, Shanghai, China). Briefly, SGC7901 cells were infected with the virus for 24 h, and the cells were then detached and plated on 0.3% agarose with a 0.5% agarose underlay in 6-well plates (1.0×10⁴ cells/well). The number of foci (>100 μm) was counted after 17 days. Each experiment was performed in triplicate.

Reverse-transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. One microgram of RNA was subjected to reverse transcription. The PCR primers used were as follows: for KLF8, forward 5'-TCTGCAGGGACTACAGCAAG and reverse 5'-TCA CATTGGTGAATCCGTCT, and for GAPDH, forward 5'-TGGTATCGTGGGAAGGACTCA and reverse 5'-CCAGTA GAGGCAGGGATGAT. PCR products were separated on a 1% agarose gel and visualized and photographed under ultra-violet light.

Western blot analysis

After protein quantization using a Coomassie brilliant blue assay, 50 μg of protein was boiled in loading buffer, resolved on 10% SDS-polyacrylamide gels, electrotransferred to nitrocellulose membranes, and incubated overnight with mouse monoclonal antibodies against KLF8, Bcl-2, Bax (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin D1, p27, cdk2, cdk4 and P21 (1:1,000; Cell Signaling, Danvers, MA, USA), and β-actin (1:5,000; Sigma, St. Louis, MO, USA). The secondary antibody (1:1,000; peroxidase-conjugated anti-mouse IgG) was ap-

plied, and the relative content of the target proteins was detected by chemiluminescence.

Flow cytometry analysis

Flow cytometry analysis was used to determine the distribution of the cells in the cell cycle or undergoing apoptosis and was performed as previously described [12]. Briefly, SGC7901 cells were seeded and infected with virus for 96 h in complete medium and then placed in a serum-free medium for 48 h. After adherent cells were collected by trypsinization, the cells were suspended in about 0.5 ml of 70% alcohol and kept at 4°C for 30 min. The suspension was filtered through a 50- μ m nylon mesh, and the DNA content of the stained nuclei was analyzed using a flow cytometer (EPICS XL; Beckman Coulter, Brea, CA, USA). Cell cycle analysis was performed using MultiCycle DNA cell cycle analysis software (BD Bioscience, USA). Each experiment was performed in triplicate. Apoptotic cells were identified as a hypodiploid DNA peak, which represented the cells in sub-G1. Findings from at least 20,000 cells were collected and analyzed using CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

Tumorigenicity in nude mice

Tumorigenicity in nude mice was determined as described previously [13]. Four groups of 4-week-old nude mice were subcutaneously injected with stably transfected cells at a single site. Tumor onset was scored visually and by palpitation at the sight of injection by two trained laboratory staff at different times on the same day. Average tumor size was estimated by physical measurement of the excised tumor at the time of sacrifice. With the exception of mice with large tumor burdens, animals were sacrificed 4 weeks after injection.

Immunohistochemistry

Tissue samples were fixed in a 10% buffered formalin solution and embedded in paraffin. For KLF8 immunostaining, a mouse anti-KLF8 monoclonal antibody was used at a 1:200 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The tumor sections were visualized using a streptavidin peroxidase kit (Beijing Zhongshan Company, Beijing, China). Signal was detected using 3,3-diaminobenzidine as a chromogen.

In situ apoptosis detection by TUNEL assay

The detection of apoptosis was performed according to the TACS TdT kit instruction (R&D Systems, Minneapolis, MN, USA). To strip proteins from the nuclei, tissue sections were incubated in 20 μ g proteinase K (Sigma-

Aldrich, St. Louis, MO, USA) per milliliter of 0.01 M phosphate-buffered saline (PBS; pH 7.4) for 15 min. Endogenous peroxidase activity was inactivated by covering the sections with 0.3% hydrogen peroxide in 50% methanol for 20 min at room temperature. The sections were rinsed three times with PBS for 3 min and immersed in terminal deoxynucleotidyl transferase (TdT) buffer for 5 min. TdT (0.3 U/ μ l) in TdT buffer was then added to cover the sections, and the sections were then incubated in a humidified chamber at 37°C for 60 min. In situ apoptosis assays were performed with a DeadEnd colorimetric terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling system (Promega, Madison, WI, USA), in which apoptotic cells were stained dark brown. For quantitative data, two slides were used from each mouse, and ten randomly selected fields were examined for each slide. The incidence of apoptosis was expressed as TUNEL-positive apoptotic cells/100 cells.

Statistical analysis

Each experiment was repeated at least three times. Bands from Western blot or RT-PCR analysis were quantized using Quantity One software (Bio-Rad, Hercules, CA, USA). Relative protein or messenger RNA (mRNA) levels were calculated relative to the amount of β -actin or GAPDH, respectively. The difference between means was performed with analysis of variance and subsequent post hoc tests. All statistical analyses were performed using SPSS v11.0 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

KLF8 is overexpressed in gastric cancer samples and gastric cancer cell lines

We examined the expression of KLF8 in 80 samples from patients with gastric cancer. The KLF8 expression in gastric cancer tissues was significantly higher than that in the adjacent nontumor gastric tissues, as determined by immunohistochemistry, and the representative pictures are presented in Fig. 1a, b. To further confirm these observations, we used Western blot analysis and RT-PCR, as showed in Fig. 1c, e. KLF8 expression was clearly elevated in tumor tissues as compared with adjacent nontumor gastric tissues. In cell lines, KLF8 protein expression was drastically increased in the three cancer cell lines, sgc7901, AGS, and MKN45, compared to that in the immortalized gastric epithelial mucosa cell line GES (Fig. 1d). These results indicated an association between the KLF8 overexpression and gastric cancer.

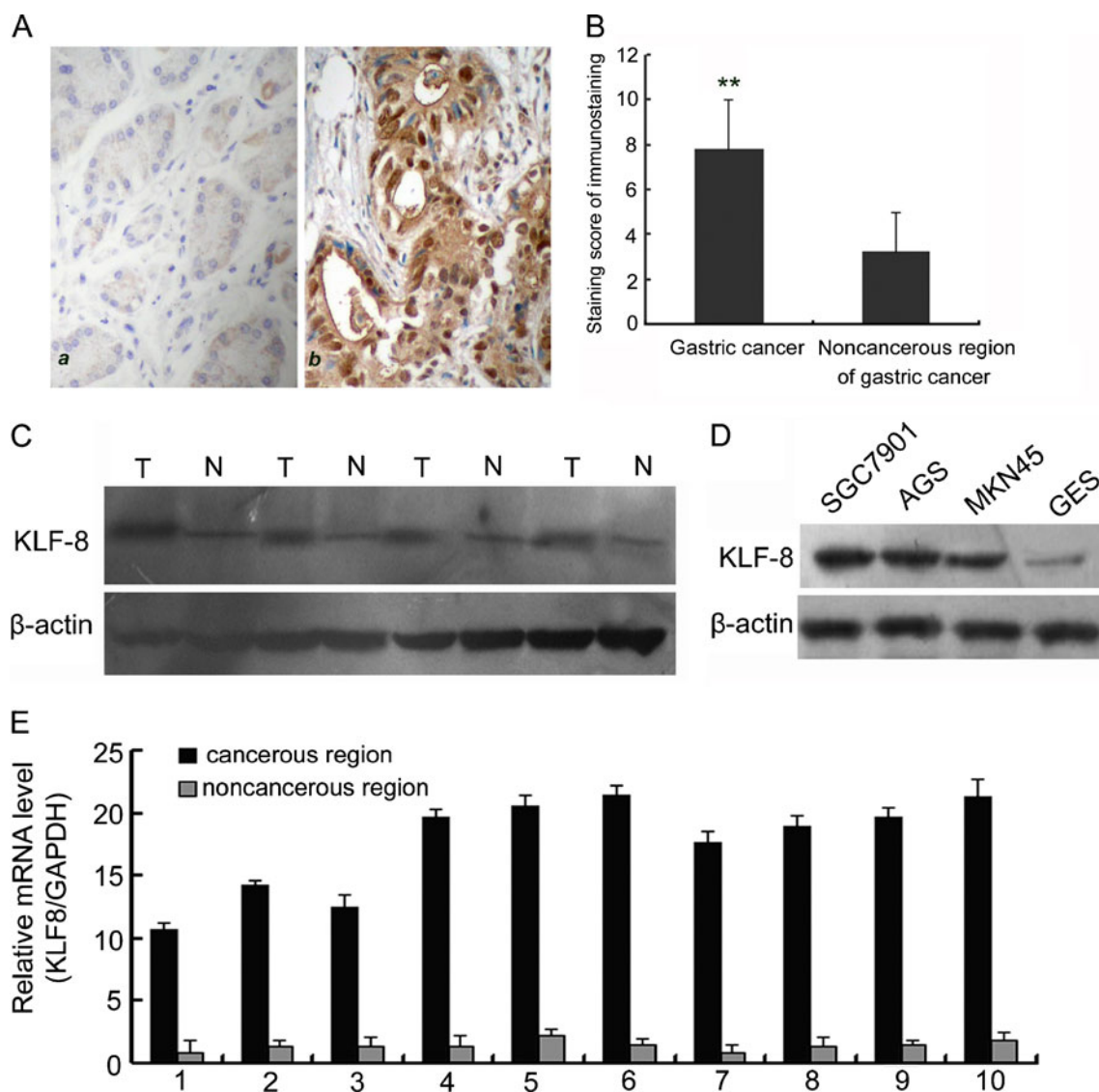


Fig. 1 Immunohistochemistry, western blot, and RT-PCR analysis of KLF8 protein and mRNA expression in gastric cancer and adjacent healthy gastric tissues. **a** KLF8 protein expression in adjacent healthy gastric tissues and gastric cancer tissues ($\times 400$). **b** Immunostaining scores in the gastric cancer group are higher than that in the adjacent healthy tissue group. **c** Western blot analysis of whole-cell protein

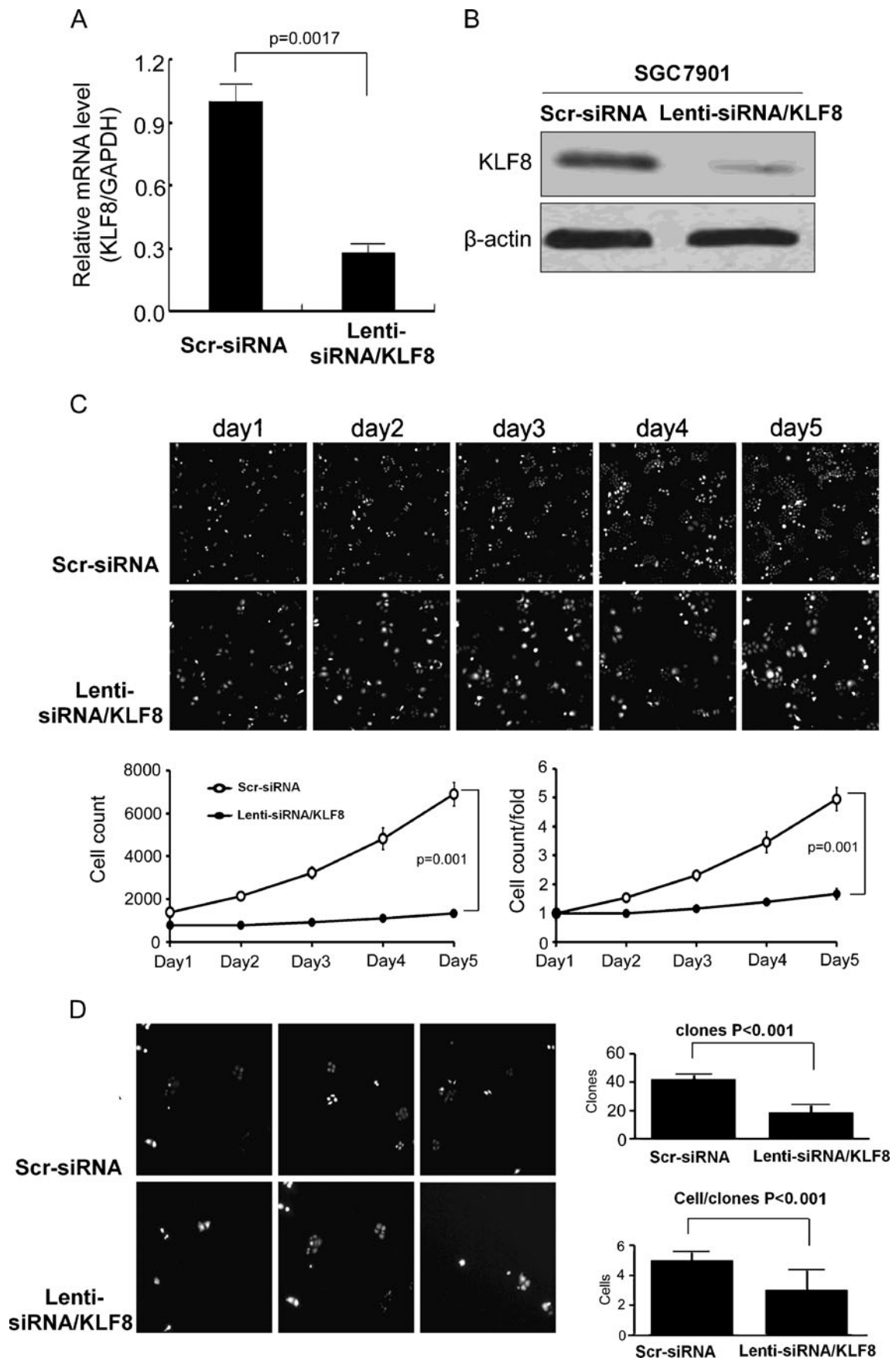
extracts prepared from four paired adjacent healthy gastric (N) and gastric cancer tissues (T). **d** KLF8 protein expression is dramatically increased in gastric cancer cell lines compare with the immortalized GES gastric epithelial mucosa cell line. β -actin expression levels were used as internal controls. **e** Gastric tissues had higher KLF8 mRNA levels than adjacent healthy gastric tissues ($P < 0.01$)

Lentivirus-delivered siRNAs knockdown KLF8 expression and inhibit SGC7901 cell proliferation

To further explore the relationship between KLF8 and gastric cancer, we constructed two lentivirus-delivered vectors: a KLF8-specific siRNA vector (lenti-siRNA/KLF8) and a scramble siRNA vector (src-siRNA). The two vectors were then transfected into SGC7901 cells. First, RT-PCR and Western blot analyses showed that KLF8 expression in lenti-siRNA/KLF8-transfected cells was much lower than that in scr-RNA-transfected cells (Fig. 2a, b). These results confirmed that lenti-siRNA/KLF8 efficiently repressed KLF8 expression. We then

tested the effect of lenti-siRNA/KLF8 on the cell viability of SGC7901 cells in culture. As shown in Fig. 2c, lenti-siRNA/KLF8 significantly inhibited cell

Fig. 2 Lentivirus vectors for the KLF8 siRNA were constructed and shown to be specific and potent for silencing KLF8 expression in the SGC7901 gastric cell line. **a** Real-time PCR of KLF8 mRNA levels from SGC7901 cells infected with the lenti-siRNA/KLF8 and src-siRNA. The src-siRNA group was used as a blank control. **b** Western blot analysis of KLF8 protein expression in SGC7901 cells infected with the lenti-siRNA/KLF8 and src-siRNA. **c** Lenti-siRNA/KLF8 depressed the growth curves of SGC7901 cells as compared with the scr-siRNA, as determined using a Cellomics Arrayscan with time-dependent manner. **d** Colony formation assays showed that the lenti-siRNA/KLF8 inhibited the number of cells and clones when compared with the scr-siRNA



growth of SGC7901 cells as compared with scr-siRNA-transfected cells. The difference was more pronounced with time-dependent manner. Colony formation assays also showed the colony-forming ability of SGC7901 was significantly inhibited by transfection with lenti-siRNA/KLF8 (Fig. 2d). These results demonstrated that knockdown of KLF8 by lenti-siRNA/KLF8 could inhibit the proliferation of SGC7901 cells compared with the scr-siRNA-transfected cells.

Lenti-siRNA/KLF8 inhibits cell cycle S-phase entry of SGC7901 cells

We performed flow cytometry at 96 h after infection. The percentage of SGC7901 cells in the G0/G1 phase in the lenti-siRNA/KLF8-transfected group (78.1 ± 3.1) was much higher than that in the scr-siRNA-transfected group (52.3 ± 2.3 ; $p < 0.01$). In addition, compared with the scr-siRNA-transfected group (32.2 ± 1.6), there was an expected decrease in the percentage of SGC7901 cells in the S phase in the lenti-siRNA/KLF8-transfected group (9.1 ± 2.5 ; $p < 0.01$), as shown in Fig. 3a. Taken together, these data indicated that lenti-siRNA/KLF8 exhibited a specific inhibitory effect on SGC7901 cell growth as well as induction of a G0/G1 phase arrest and inhibition of S-phase entry.

In order to explore the underlying molecular mechanism of how KLF8 affects the cell cycle distribution, we detected the expression of cell cycle-related molecules in lenti-siRNA/KLF8-transfected SGC7901 cells, such as cyclin D1, p27, cdk2, cdk4, and p21, by Western blot analysis. The results of these experiments found that the expression of cdk2, cdk4, and p21 were not affected (data not show) and that cyclin D1 expression was downregulated and p27 was upregulated (Fig. 3b). Therefore, we proposed that G1 to S arrest induced by knockdown of KLF8 in SGC7901 cells was at least partly mediated by downregulation of cyclin D1 and upregulation p27 expression.

Lenti-siRNA/KLF8 induces cell apoptosis in SGC7901 cells

The effect of lenti-siRNA/KLF8 on the apoptosis of SGC7901 cells was investigated by flow cytometry (Fig. 3c). It was found that 11.65% of the SGC7901 cells transfected with scr-siRNA were observed to be apoptotic, while significantly more apoptotic cells were observed in those transfected with lenti-siRNA/KLF8 (24.43%). These data suggested that knockdown of KLF8 by lenti-siRNA/KLF8 specifically induced apoptosis of the KLF8-overexpressing gastric cancer cell line SGC7901.

In order to explore the underlying molecular mechanism of how KLF8 affected cell apoptosis, we analyzed the Bcl-2

family mediators of apoptosis, such as Bcl-2 and Bax, via Western blot analysis. The results indicated that knockdown of KLF8 downregulated Bcl-2 and upregulated Bax expression in lenti-siRNA/KLF8-transfected cells, suggesting that the apoptotic effect of KLF8 may be partly mediated by these Bcl-2 family proteins (Fig. 3d).

Lenti-siRNA/KLF8 inhibits tumor growth in vivo

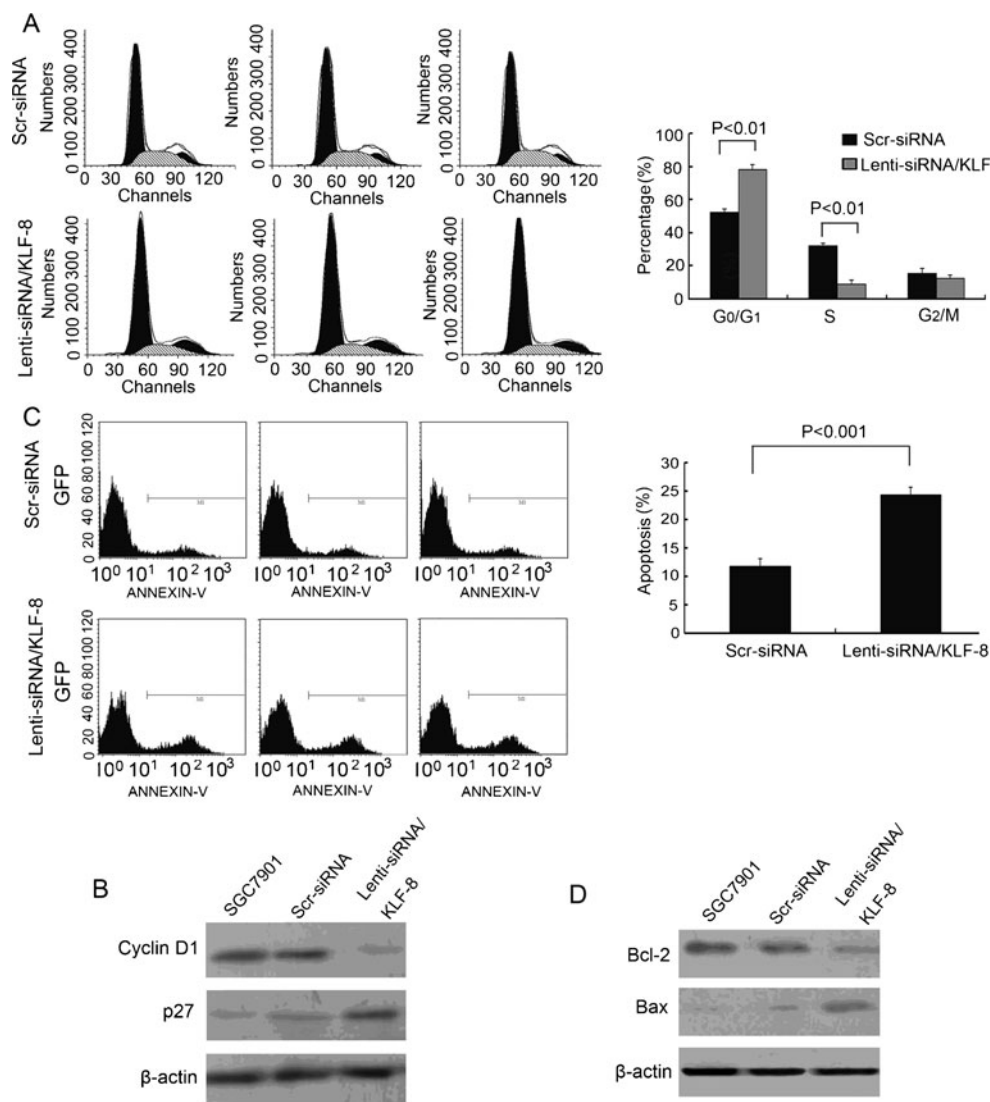
To determine whether lenti-siRNA/KLF8 could serve as a therapeutic agent against gastric cancer formation, we adopted a subcutaneous tumor formation assay. Mice were subcutaneously injected with stably transfected cells at a single site. After 2 weeks, the tumor growth in the lenti-siRNA/KLF8-transfected cell group was expectedly inhibited as compared with the scr-siRNA-transfected cell group (Fig. 4a, b). Immunohistochemical analysis showed that KLF8 protein expression in the lenti-siRNA/KLF8-transfected cell group was decreased compared to that in the other groups (Fig. 4c). This result confirmed that lenti-siRNA/KLF8 suppressed KLF8 expression in vivo. The specimens were then subjected to in situ apoptosis detection via TUNEL staining. As shown in Fig. 4d, the lenti-siRNA/KLF8-transfected cell group demonstrated dramatically induced apoptotic cell death, whereas the scr-siRNA-transfected cell group and IgG control group did not demonstrate any tumor cell apoptosis. Taken together, these data indicated that targeting KLF8 with lenti-siRNA/KLF8 could have an inhibitory effect in vivo on gastric cancer in which KLF8 is overexpressed and inhibit tumor growth by triggering cell apoptosis.

Discussion

Gastric cancer is one of the most common cancers worldwide, and current clinical management is mostly unsatisfactory due to the high rate of recurrence and the aggressive behavior of this cancer [1, 2]. With the expectation of improving therapeutic efficacy, gene therapy is being studied as a possible therapeutic modality [14]. The tumorigenesis and progression of gastric cancer are far from being fully elucidated. Hence, identification of factors associated with tumor progression and recurrence and their underlying mechanisms are especially important [15].

Overexpression of KLF8 has been recently reported in several types of human malignancies, including ovarian [8], breast cancer [9], and renal cell carcinoma [10]. In this study, we determined that KLF8 expression is significantly increased in gastric cancer tissues and gastric cancer cell lines compared with adjacent nontumor gastric tissues and GES cells. This result suggested an association between the overexpression of KLF8 and gastric cancer.

Fig. 3 The effects of lenti-siRNA/KLF8 on cell cycle and cell apoptosis as determined by flow cytometric analysis. **a** Flow cytometry analysis showed that knockdown of KLF8 expression via the lenti-siRNA/KLF8 induced G0/G1 phase arrest and decreased the S phase population of the cells, indicating disruption of cell cycle progression. **c** FCM showed that knockdown of KLF8 expression via lenti-siRNA/KLF8 increased cell apoptosis. **b** Cell cycle-related molecules were assayed via western blot analysis. Knockdown of KLF8 expression by lenti-siRNA/KLF8 resulted in downregulation of cyclin D1 and upregulation of p27 expression in SGC7901 cells. **d** Western blot analysis of Bcl-2 family mediators of apoptosis showed that knockdown of KLF8 resulted in downregulation of Bcl-2 and upregulation of Bax in lenti-siRNA/KLF8-transfected cells, suggesting that the apoptotic effect of KLF8 might be partly mediated by Bcl-2 family member proteins



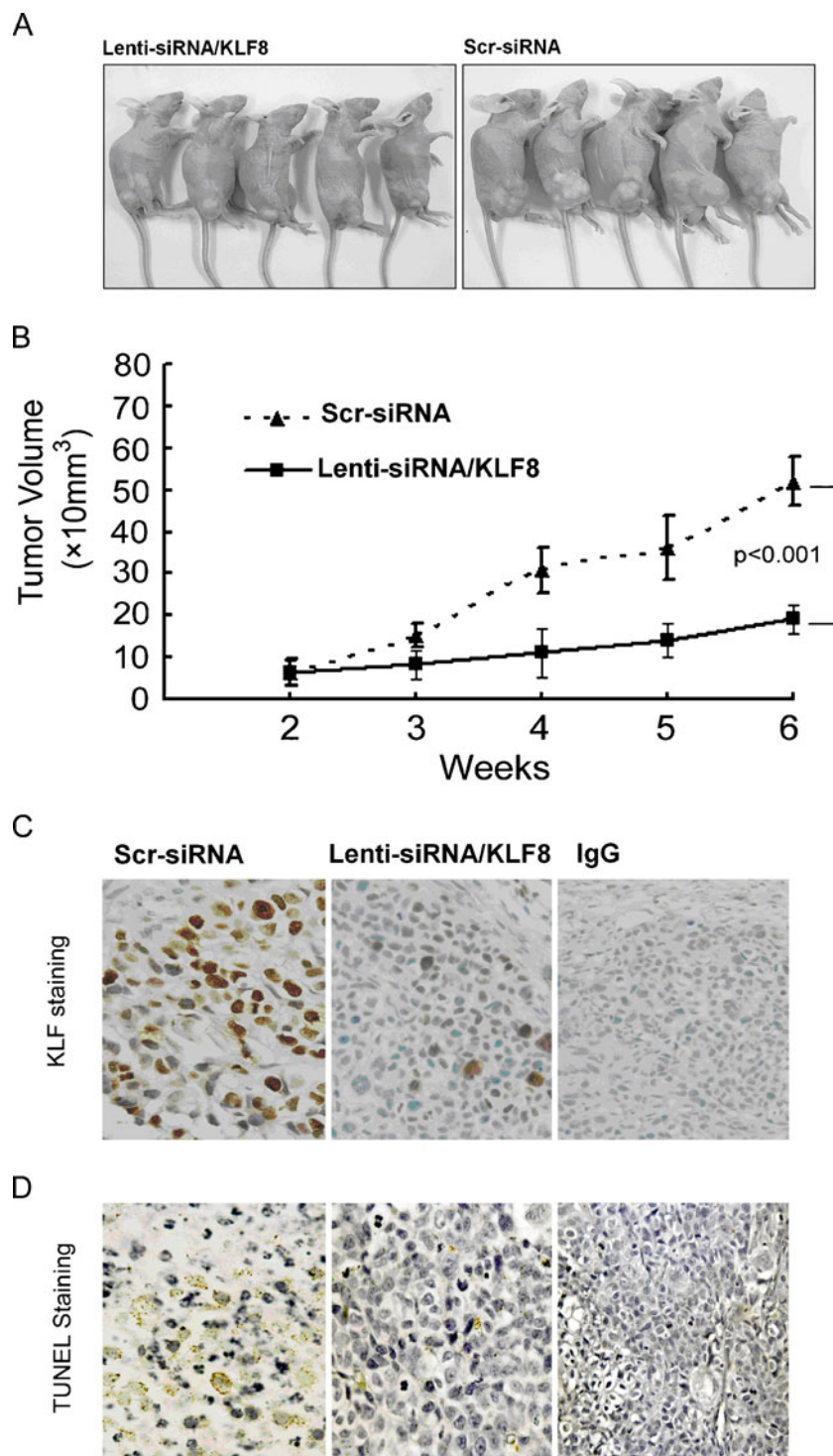
Lentivirus is an efficient gene delivery vector because of its capability to deliver a large number of genetic messages into the host cell DNA and replicate in nondividing cells [16]. We used a lentivirus vector in our system to infect and efficiently silence KLF8 in the SGC7901 gastric cancer cell line.

In this study, we constructed the lenti-siRNA/KLF8 lentivirus vector, which efficiently knocked down the expression levels of the KLF8 mRNA and protein and inhibited the proliferation of and induced apoptosis in SGC7901 cells compared with the control src-siRNA vector. Furthermore, lenti-siRNA/KLF8 inhibited gastric cancer growth in vivo, as tumor volumes were significantly suppressed when lenti-siRNA/KLF8-transfected SGC7901 cells were injected into nude mice.

Tumors acquire invasive phenotypes via EMT, which is mediated by many factors, including focal adhesion kinase (FAK), that play critical roles in tumor growth,

initiation, and metastasis [17–19]. Recently, KLF8 has been reported as a crucial transcription factor downstream of FAK, which is involved in mediating cell cycle progression via upregulation of cyclin D1 [20]. We observed a significantly decreased S-phase population in lenti-siRNA/KLF8-transfected SGC7901 cells, and this blockage to S-phase entry was connected with the downregulation of cyclin D1 and upregulation of p27 expression. This result indicated that knockdown of KLF8 expression inhibited gastric cancer cell proliferation regulated by cyclin D1 and p27 to activate expression of genes necessary for entering into the S phase. A recent report demonstrated that EMT may effect tumor proliferation by promoting tumorigenicity and cell cycle arrest, and it has been reported that KLF8 overexpression promotes EMT in breast cancer [6]. However, this transformation and its role in gastric cancer cell proliferation still require further study.

Fig. 4 Local injection of lenti-siRNA/KLF8-transfected SGC7901 cells into mice resulted in clear inhibition of tumor growth in vivo. **a** The tumor size of the lenti-siRNA/KLF8-transfected cell group was significantly decreased compared to that of the scr-siRNA-transfected cell group. **b** Tumor growth curves showed a significant growth tendency in the scr-siRNA-transfected cell group, while tumor growth in the lenti-siRNA/KLF8-transfected cell group was clearly inhibited ($p < 0.001$). **c** KLF8 protein expression in the lenti-siRNA/KLF8-transfected cell group decreased in vivo compared to that in the other groups, as determined by immunohistochemistry; IgG was used as an internal control. **d** TUNEL staining showed that in vivo cell apoptosis in the lenti-siRNA/KLF8-transfected cell group was clearly increased compared to that in the other groups; IgG was used as an internal control



In addition, knockdown of KLF8 induced apoptosis in SGC7901 cells. Previous studies revealed that the expression of Bcl-2 family proteins was associated with cell growth and apoptosis in SGC7901 cells [21]. Our study found that depletion of KLF8 could downregulate Bcl-2 and upregulate Bax, demonstrating that Bcl-2 family members may contribute to the apoptosis of gastric cancer

cells following knockdown of KLF8. We also demonstrated that local injection of lenti-siRNA/KLF8-transfected SGC7901 cells into nude mice resulted in significantly inhibited in vivo tumor growth via induction of apoptosis, as detected by TUNEL staining. These results suggested that KLF8 overexpression may be essential for maintaining cell proliferation and survival as well as inhibition of cell

apoptosis in gastric cancer cells. Our ongoing study further validates the anti-apoptosis role of KLF in other gastric cancer cell lines.

In conclusion, the results of this study suggested that the significant downregulation of KLF8 expression by lenti-siRNA/KLF8 in gastric cancer cells resulted in inhibited cell proliferation both in vitro and in vivo and induced cell apoptosis. Therefore, knockdown of KLF8 by lentivirus-delivered siRNA may be a valuable approach for the treatment of gastric cancer in which KLF8 is overexpressed.

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Conflicts of interest None.

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