

Lentivirus-mediated RNA interference targeting *EBNA1* gene inhibits the growth of GT-38 cells *in vitro* and *in vivo*

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Abstract. Epstein-Barr virus nuclear antigen 1 (*EBNA1*) is associated with the pathogenesis of Epstein-Barr virus-associated gastric carcinoma (EBVaGC). However, the function of *EBNA1* in the growth of EBVaGC cells remains unclear. In the present study, the effects of silencing *EBNA1*, by RNA interference (RNAi), on the growth of EBVaGC cells were investigated *in vitro* and *in vivo*. A lentivirus-mediated RNAi targeting *EBNA1* was transfected into the EBVaGC cell line GT-38. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR), western blot analysis, MTT, colony formation and flow cytometry were performed to evaluate the biological behavior of GT-38 cells that were transfected with *EBNA1* small interfering RNA (siRNA) *in vitro*. The effects of silencing *EBNA1* on tumor growth were assessed in a tumor xenograft model using BALB/c nude mice. The results demonstrated that the proliferative and clonogenic abilities of GT-38 cells were significantly downregulated in response to *EBNA1* siRNA ($P < 0.01$). Furthermore, *EBNA1* siRNA induced cell cycle arrest in the G₀/G₁ phase and promoted apoptosis of GT-38 cells ($P < 0.01$). The tumorigenicity of GT-38 cells was significantly inhibited in the *EBNA1* siRNA group. The results revealed that lentivirus-mediated RNAi of *EBNA1* inhibited the growth of the EBVaGC cell line GT-38 *in vitro* and *in vivo*. Therefore, *EBNA1* may be a potential target for gene therapy in EBVaGC.

Introduction

Epstein-Barr virus (EBV) is the first human tumor virus to be identified, affecting >95% of the human population worldwide. EBV establishes a life-long persistent infection, causing a variety of human malignancies, including Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma (NPC) and

gastric cancer (GC) (1). EBV-associated (EBVaGC) accounts for ~7.5-10% of gastric carcinoma cases, which is estimated to exceed 75,000 cases/year (2,3). EBV is involved in the pathogenesis of EBVaGC. EBV is present in all tumor cells but not in the surrounding normal epithelium. EBVaGC exhibits unique morphological features (4). Further research on the key genes of EBV may provide novel targets for the treatment of EBVaGC.

EBVaGC cells express a well-defined set of latent genes including Epstein-Barr virus nuclear antigen 1 (*EBNA1*), EBV-encoded small RNA and latent membrane protein 2A. *EBNA1* is a viral antigen expressed in all EBVaGC cells, and there are an increasing number of studies, which suggest that *EBNA1* may alter the cellular environment and promote genomic instability, therefore leading to tumorigenesis (5-7). To the best of our knowledge, a limited number of studies have utilized EBV-positive gastric carcinoma cells containing a wild-type *EBNA1* gene (8), including the GT-38 cell line.

The present study aimed to investigate the biological function of *EBNA1* in gastric carcinoma. Gene silencing by RNA triggers an evolutionarily conserved process known as RNA interference (RNAi) (9). Lentiviral vectors have emerged as powerful vectors for gene transfer (10). Therefore, several experiments have been conducted to detect whether *EBNA1* suppression could inhibit GT-38 cell proliferation *in vitro* and *in vivo*.

Materials and methods

Cell line and culture condition. EBVaGC cell line GT-38 and 293 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Excell Bio, Shanghai, China), 50 U/ml penicillin and 50 U/ml streptomycin (both from Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere containing 5% CO₂. The medium was changed every 2 days and cells were passaged every 4-5 days.

Lentiviral infection. Small interfering RNAs (siRNAs) targeting the *EBNA1* sequence were purchased from Dharmacon. The siRNA sequences for *EBNA1* were: 3'-GGAGGTTCCAAC CCGAAAT-5'. A non-silencing siRNA was used as a negative control (cat. no. D001810-01-20). One day before transfection, a total of 1x10⁵ GT-38 cells in the logarithmic growth phase were

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seeded in 6-wells plates at 37°C in RPMI-1640 (10% FBS). 200 nM siRNA and 5 μ l Lipofectamine 200 (Thermo Fisher Scientific, Inc.) were mixed in fresh RPMI-1640 (without FBS) and incubated for 20 min at room temperature. When the cell reached 80-90% confluence, the media was replaced with fresh RPMI-1640 (without FBS) and the mixtures was dropwise addition to cells. After 8 h, the media was replaced with fresh RPMI-1640 (supplemented with 10% FBS). At 48 h later, the cells were split for subsequent experiments. Three groups were used in the *in vitro* experiments, including untreated cells (Blank control group), cells transfected with non-silencing siRNA (Control siRNA group) and cells transfected with the *EBNA1* siRNA (*EBNA1* siRNA group).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). GT-38 cells were harvested after infection. Total RNA was isolated from GT-38 cells using TRIzol (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA was transcribed into cDNA using the cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). The cDNA was amplified using the SYBR-Green PCR Master Mix (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. The thermocycling conditions were as follows: Initial denaturation of 95°C for 1 min followed by 40 cycles of 95°C for 5 sec and final extension of 60°C for 20 sec. The following primers were used: *EBNA1* forward, 5'-CGCATCATAGACCGCCAGTA-3' and reverse, 5'-CTG GCCCCTCGTCAGACAT-3'; *GAPDH* forward, 5'-CTCAGA CACCATGGGGAAGGTGA-3' and reverse, 5'-ATGATC TTGAGGCTGTTGTCATA-3'. The reactions were performed in triplicate. The relative expression of target gene was evaluated using the $2^{-\Delta\Delta C_q}$ method with *GAPDH* as the internal reference (11).

Western blot analysis. Protein lysates from cells were harvested using a radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China). The concentration of protein was determined using a bicinchoninic acid assay (Beyotime Institute of Biotechnology). Equal amounts of protein (10 μ g) were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h. Following blocking, the membranes incubated overnight at 4°C with the following primary antibodies: Anti-*EBNA1* (cat. no. sc-81581, 1:10,000) and anti-*GAPDH* (cat. no. sc-66163, 1:10,000) (both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse with secondary antibodies for 1 h at room temperature (cat. no. sc-2005, 1:3,000; Santa Cruz Biotechnology, Inc.). The bands were detected using enhanced chemiluminescence (Thermo Fisher Scientific, Inc.). Densitometric analysis was performed using Quantity One 4.6.9 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of three independent experiments were performed.

MTT assay. Following lentivirus infection, the viability of GT-38 cells was evaluated using a MTT assay at 24, 48 and 72 h. The cells were seeded in 96-wells plates at a cell density

of 3,000 cells/well in 200 μ l RPMI-1640. MTT solution was added into each well, and the cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 4 h. Next, 150 μ l acidic isopropanol (0.01 M HCl, 10% SDS, 5% isopropanol) was added in each well to stop the reaction. The absorbance was assessed at 450 nm using a microplate reader (Epoch; Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of 5 parallel MTT experiments were performed for each group. The experiments were performed in triplicates.

Colony formation assay. Following lentivirus transfection, GT-38-transfected cells were seeded into 6-cm dishes at a density of 8x10² cells/dish and cultured in RPMI-1640 at 37°C for 2 weeks. Then, the cells were fixed with 4% paraformaldehyde for 15 min, stained with Giemsa (both from Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 20 min and washed twice with ddH₂O. Visible colonies containing ≥ 50 cells were manually counted under fluorescence microscopy (Olympus Corporation, Tokyo, Japan). Image analysis was performed using Image-Pro® Plus (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA). A total of 5 parallel experiments were performed for each group. The experiments were performed in triplicates.

Cell cycle analysis. Following lentivirus infection, GT-38 cells were seeded in 6-cm dishes at 5x10⁴ cells/dish and incubated at 37°C for 48 h. The cells were harvested using 0.25% trypsin-EDTA and fixed with 70% ice-cold ethanol for 2 h. Then, the cells were resuspended in 0.1% sodium citrate, 0.1% Triton X-100 and 20 mg/ml propidium iodide (PI; BD Biosciences, Franklin Lakes, NJ, USA) for 1 h in the dark. At least 10,000 events were collected for each sample. The cells were analyzed using a flow cytometer (FACSCalibur™; BD Biosciences) and the results were analyzed using ModFit 3.0 software (Verity Software House, Inc., Topsham, ME, USA). Five parallel were performed for each group. The experiments were performed in triplicates.

Apoptosis assay. At 48 h post-transfection, a total of 1.0x10⁶ cells were collected and stained with Annexin V-fluorescein isothiocyanate (FITC; BD Biosciences) and PI according to the manufacturer's protocol. Briefly, 1x10⁶ cells were washed with 1X binding buffer and centrifuged at 300 x g for 10 min. The cell pellet was resuspended in 100 μ l 1X binding buffer. Then, 10 μ l Annexin V-FITC/10⁶ cells were added, mixed and incubated for 15 min in the dark at room temperature prior to analysis by a flow cytometer. Five parallel were performed for each group. The experiments were performed in triplicate.

Tumor xenograft model. A total of 6 SPF male BALB/c nude mice (age, 4-6 weeks; weight, 20-24 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China), and were maintained inverse 12 light/dark cycle had free access food and water at room temperature under a specific pathogen-free condition. GT-38 cells (2x10⁶ cells/tumor) were injected subcutaneously into the left axilla of nude mice. When the xenografts reached a size of approximately 0.5 cm in diameter, the mice were randomly divided into two groups (the *EBNA1*-siRNA and blank control group, 3 mice per

group). Then the mice were injected with *EBNA1*-RNAi and Lipofectamine or Lipofectamine alone every 2 days for a total of 2 weeks. At 2 weeks of treatment, the mice were sacrificed and the tumors were harvested. All animal experiments in the present study were approved by the Ethics Committee of Linyi People's Hospital (Shandong, China).

Statistical analysis. All Statistical analysis were conducted using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). The relevant data are expressed as the mean \pm standard deviation (SD). One-way analysis of variance followed by Bonferroni's post hoc test was used to examine differences among multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

***EBNA1* siRNA downregulates *EBNA1* expression in GT-38 cells.** The GT-38 cell line was selected to investigate the function of *EBNA1* in EBVaGC. GT-38 cells were transfected using a lentiviral vector system expressing siRNA that targets *EBNA1*. RT-qPCR and western blot analysis revealed that mRNA and protein levels of *EBNA1* exhibited a 52 and 42.7% reduction, respectively, in response to treatment with *EBNA1*-siRNA compared with the control-siRNA or blank control groups (Fig. 1A-C). These results indicated an efficient downregulation of *EBNA1* using the lentiviral vector delivery system.

Knockdown of EBNA1 suppresses the viability and proliferation of GT-38 cells. The effects of knocking down *EBNA1* on the viability and proliferation of GT-38 cells was evaluated using a MTT and colony forming assay. The results demonstrated that the knockdown of *EBNA1* decreased the viability of GT-38 cells by 65% when compared with the blank control group ($P < 0.01$; Fig. 2A). Furthermore, colony formation assay demonstrated that the knockdown of *EBNA1* resulted in smaller and fewer colonies compared with the control siRNA and blank control groups ($P < 0.01$; Fig. 2B and C). These results suggested *EBNA1* might inhibit proliferation and suppress the clonogenic potential of GT-38 cells.

Knockdown of EBNA1 induces cell cycle arrest in the G₀/G₁ phase in GT-38 cells. Flow cytometric analysis of cell cycle distribution of *EBNA1* siRNA-infected GT-38 cells was performed to examine how *EBNA1* promotes the proliferation and colony formation potential of GT-38 cells. The results demonstrated a significant increase in the percentage of cells in the G₀/G₁ phase and a decreased number of cells in the G₂/M phase ($P < 0.01$) in response to treatment with *EBNA1* siRNA (Fig. 3). However, no differences in cell numbers were observed in the S phase in the *EBNA1* siRNA group compared with the control siRNA and blank control groups (Fig. 3). These results suggested that down regulating *EBNA1* might induce cell cycle arrest in the G₀/G₁ phase.

Knockdown of EBNA1 induces the apoptosis of GT-38 cells. The effects of downregulating *EBNA1* on the apoptosis of GT-38 cells was examined by Annexin V/PI double staining and flow cytometry. The results demonstrated that

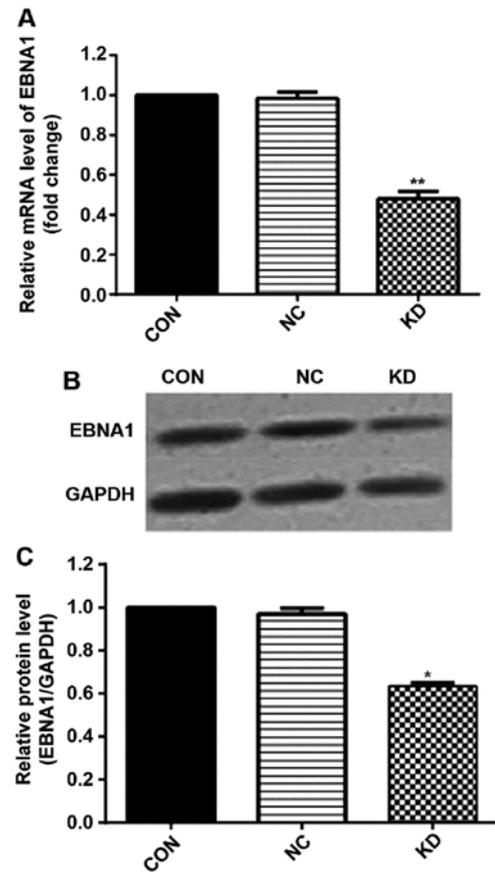


Figure 1. *EBNA1*-siRNA downregulates the expression levels of *EBNA1* in GT-38 cells. (A) *EBNA1*-siRNA downregulated the mRNA expression levels of *EBNA1* in GT-38 cells. ** $P < 0.01$, $n = 5$. (B) *EBNA1*-RNAi-LV downregulated the protein expression levels of *EBNA1* in GT-38 cells. (C) Densitometric analysis of western blots from three independent experiments, respectively. β -actin was used as internal controls. * $P < 0.05$ vs. the blank control groups. EBNA1, Epstein-Barr virus nuclear antigen 1; siRNA, small-interfering RNA.

the downregulation of *EBNA1* promoted apoptosis (Fig. 4). The percentage of early and late apoptotic cell population increased by 11.8 and 21.1% in the *EBNA1*-siRNA group compared with the control-siRNA and blank control groups, respectively ($P < 0.01$; Fig. 4). These results indicated that the knockdown of *EBNA1* might promote cell apoptosis in EBVaGC cells.

Knockdown of EBNA1 inhibits the growth of GT-38 cells in vivo. To study the *in vivo* tumorigenic potential of EBNA1, GT-38 cells were inoculated under the skin of nude mice and the mice were injected with *EBNA1*-siRNA with lipofectamine or lipofectamine alone. After 2 weeks, tumor development was observed in the control group (Fig. 5A). The median tumor weight was significantly decreased by >79.7% ($P < 0.001$; Fig. 5B) in the *EBNA1*-siRNA group, suggesting that downregulating EBNA1 may inhibit the growth GT-38 cells *in vivo*.

Discussion

Tumor viruses account for 50% of cases of human cancer (4), which may be increasing as novel human tumor viruses are being identified. In order to identify these types of

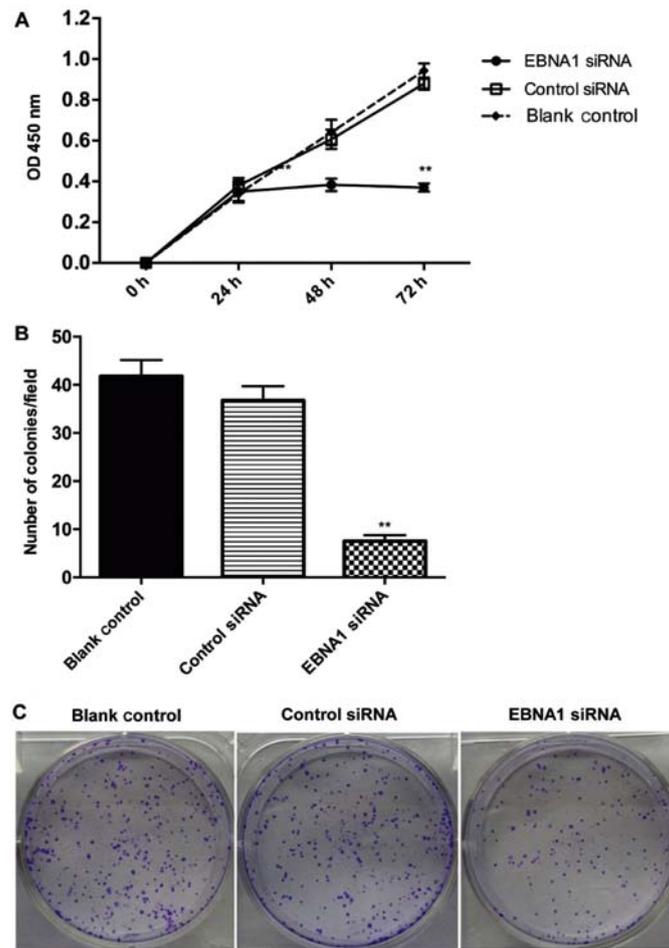


Figure 2. Knockdown of *EBNA1* suppresses the viability and proliferation of GT-38 cells. (A) Cell proliferation was determined using a MTT assay. A total of 5 parallel MTT assays were performed for each group. The experiments were performed in triplicates. (B) Cell proliferation in blank, control-siRNA and *EBNA1*-siRNA groups was evaluated using the colony formation assay. (C) Representative images of colonies in the blank, control-siRNA and *EBNA1*-siRNA groups. A total of three independent experiments were performed. ** $P < 0.01$ vs. the blank control groups. EBNA1, Epstein-Barr virus nuclear antigen 1; siRNA, small-interfering RNA; OD, optical density.

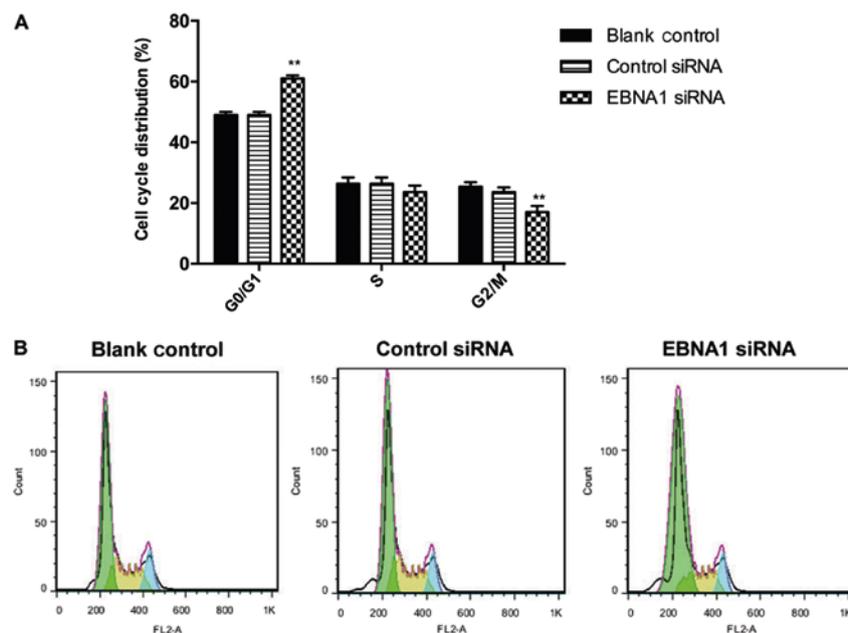


Figure 3. Knockdown of *EBNA1* induces G₀/G₁ phase arrest in GT-38 cells. (A) The proportion of cells in the G₀/G₁ phase was significantly increased, whereas the proportion of cells in the G₂/M phase was decreased in the *EBNA1*-siRNA group compared with the control siRNA and blank control groups. Three individual experiments were performed. ** $P < 0.01$ vs. the blank control groups (B) Flow cytometric analysis demonstrated *EBNA1*-RNAi induced cell cycle arrest in the G₀/G₁ phase in GT-38 cells. EBNA1, Epstein-Barr virus nuclear antigen 1; siRNA, small-interfering RNA; RNAi, RNA interference.

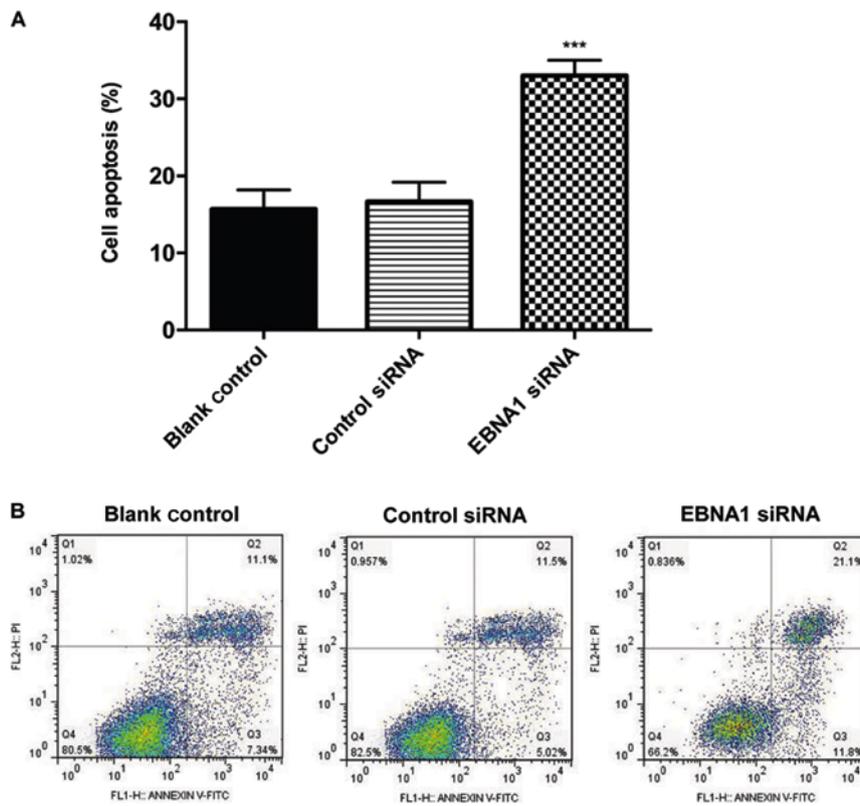


Figure 4. Knockdown of *EBNA1* promotes the apoptosis of GT-38 cells. (A) Flow cytometric analysis of apoptosis at 48 h post-transfection with *EBNA1*-siRNA. *EBNA1*-RNAi significantly promoted cell apoptosis. Three individual experiments were performed. *** $P < 0.001$ vs. the blank control group. (B) Representative images of Annexin V/PI double staining and flow cytometric analysis of apoptosis in response to treatment with *EBNA1*-siRNA. The cells in the lower left, lower right, upper right and upper left quadrant correspond to viable, early apoptotic, late apoptotic or necrotic cells, respectively. EBNA1, Epstein-Barr virus nuclear antigen 1; siRNA, small-interfering RNA; RNAi, RNA interference; FITC; fluorescein isothiocyanate; PI, propidium iodine.

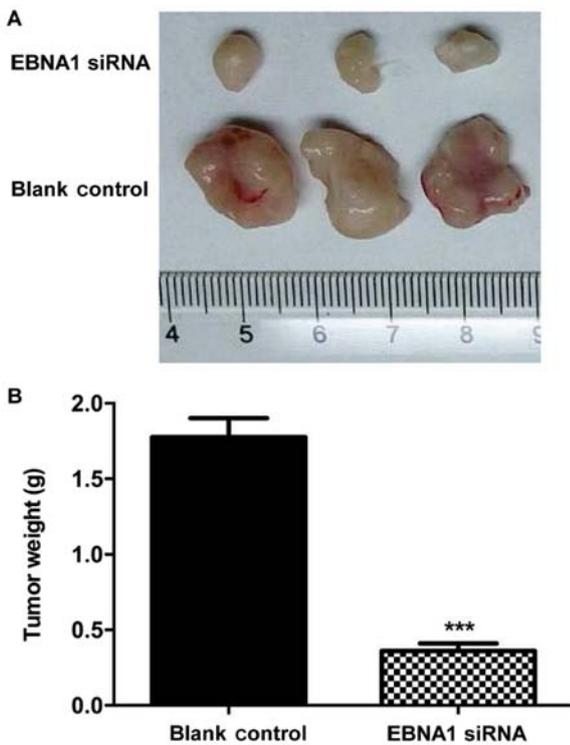


Figure 5. Knockdown of *EBNA1* inhibits the growth of GT-38 cells *in vivo*. (A) Images of tumors that were extracted after 2 weeks. (B) Tumor weights were significantly decreased in response to treatment with *EBNA1*-siRNA. $n = 3$ mice per group; *** $P < 0.001$ vs. the blank siRNA group. EBNA1, Epstein-Barr virus nuclear antigen 1; siRNA, small-interfering RNA.

virus-associated cancer, it is essential to examine the viral ability to maintain tumor phenotypes; therefore providing a non-host target for therapeutic interventions. EBV has been associated with the pathogenesis of GC (12). The present study investigated the underlying molecular mechanisms by which EBV might contribute to the development of EBVaGC that is mediated by *EBNA1*.

EBNA1 has been considered to be a novel biomarker of human GS (13). The results of the present study demonstrated that transfection with a specific siRNA that targets *EBNA1* decreases the mRNA and the protein levels of *EBNA1* by 52 and 42.7%, respectively. Ian *et al* (14), demonstrated that the silencing of *EBNA1* leads to decreased proliferation and apoptosis of EBV-positive natural killer (NK)/T cell lymphoma cells. The results of the present study demonstrated that the proliferation of EBVaGC cells was significantly decreased by 65% in response to treatment with *EBNA1*-siRNA. Sivachandran *et al* (15) demonstrated that *EBNA1* causes the disruption of promyelocytic leukemia (PML) nuclear bodies (NBs) in nasopharyngeal carcinoma (NPC) cells. A previous study demonstrated that human adenocarcinoma cell lines that contain an integrated *EBNA1* expression cassette exhibited a decreased activation of p53 and cell apoptosis as well as an increased survival rate (16). Additionally, the knockdown of *EBNA1* might decrease the proliferative and colony formation abilities of SKOV3 ovarian cancer cells (17), Burkitt's lymphoma Raji cells (18) and NPC cells (19) by inducing G_0/G_1 phase arrest. The results of the present study

demonstrated that the downregulation of *EBNA1* negatively regulated the progression of gastric cancer via induction of cell cycle arrest and late apoptosis in GT-38 cells. Our results are consistent with the results from the aforementioned studies.

To investigate the effects of *EBNA1* on the proliferation of gastric cancer cells *in vivo*, BALB/c nude mice were inoculated with GT-38 cell tumors that were treated with continued intratumoral injection of *EBNA1* siRNA. The results demonstrated that silencing *EBNA1* downregulated tumor weight by 79.7% in the gastric cancer tumor xenografts compared with the control group. These results indicated that downregulation of *EBNA1* by RNAi significantly inhibited the growth of GT-38 gastric cancer cells *in vitro* and *in vivo*.

The molecular mechanisms by which *EBNA1* may facilitate viral replication and maintain viral episome are unclear. Saridakis *et al* (20), demonstrated that *EBNA1* might decrease the levels of p53 by sequestering the ubiquitin-specific protease 7N. Sivachandran *et al* (4) demonstrated that *EBNA1* caused the disruption of PML NBs, which serve important functions in apoptosis, activation of p53 and tumor suppression, indicating that EBV infection of GC cells might lead to *EBNA1*-mediated disruption of PML NBs. The present study demonstrated that knocking down *EBNA1* might decrease proliferation and induce apoptosis in EBVAGC cells, which might be caused by the loss of PML NBs. However, future studies are required to confirm the results of the present study and investigate novel therapies for EBVAGC.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JW, FM, XX and YW designed and performed the experiments. JW, LL and CL interpreted the experimental results, wrote and revised the manuscript. All authors contributed to this manuscript. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All animal experiments in the present study were approved by the Ethics Committee of Linyi People's Hospital (Shandong, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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