Up-regulation of Rb by small activating RNA inhibits cell invasion and migration in gastric cancer cells

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Abstract

Background: Gastric cancer (GC) is a lethal disease that needs further investigation. Recent studies have reported that small activating RNA (saRNA), involved in a process called RNA activation, plays an important role in the development of cancer.

Methods: The expression of retinoblastoma (Rb) was detected in human GC tissues and cell lines. We designed three different saRNAs targeting the Rb gene promoter in GC cell lines. Upregulated Rb expression after transfection of the saRNAs was confirmed by PCR and western blotting. And GC cell proliferation, migration, and invasion were detected using CCK8 assays and Transwell assays.

Results: Here, we found that Rb displayed lower expression in GC tissues and cells. saRNA-3 significantly increased the expression of Rb in both SGC-7901 and MKN-28 cells. saRNA-3 significantly inhibited the proliferation of both cell types by day 3 after transfection. There was no significant difference between the negative control saRNA and mock transfection groups. saRNA-3 decreased the tumor volume and weight compared with the mock and dsControl groups. In addition, saRNA-3 transfection decreased the migratory ability of SGC-701 and MKN-28 cells.

Conclusions: Overall, Rb is a promising novel prognostic biomarker of GC and, due to its role in metastasis, a novel therapeutic target for the clinical management of invasive and metastatic GC.

Background

Gastric cancer (GC) is the fifth most common tumor and third leading cause of cancer-related mortality in the world [1]. However, in China, GC has the highest morbidity and mortality rates of all cancers [2]. Although the incidence of GC is decreasing by nearly 2% per year worldwide, the overall treatment effect has not fundamentally improved due to population growth [3]. The highly invasive behavior of GCs often leads to recurrence and metastasis, which are the main causes of death in patients [4]. GC-related genes, including oncogenes and tumor suppressor genes, are closely related to the malignant biological behavior of GC [5]. Therefore, in-depth investigation of the mechanisms of GC-related genes can provide the necessary theoretical basis for identifying new targets for GC treatment.

Small activating RNAs (saRNAs) act mainly on tumor suppressor genes, including those that negatively regulate the cell cycle, promote cell death, and inhibit tumor invasion and metastasis [6]. saRNAs upregulate the expression of such genes to promote tumor cell differentiation, increase apoptosis, and inhibit tumor cells [7]. In the process of RNA activation (RNAa), the expression of saRNA target genes is upregulated after the saRNA binds to a specific region of the promoter region or against the recognition of a tumor suppressor gene by non-coding small RNAs (microRNAs) [8]. In addition, saRNAs promote post-transcriptional upregulation of target genes via different pathways [9]. Compared with the traditional methods of increasing target gene expression, RNAa has unique advantages involving simple operation, low cost, very specific activation, and physiological regulation [10]. Furthermore, its effects persist in the cell but can be terminated by stopping the drug, and the activation effect is easily controlled.

At present, the mechanism of RNAa is unclear, but different studies have shown that saRNAs activate target genes at the transcriptional level by binding to certain regions of the promoter or to the antisense transcript of the target gene [11, 12]. Because saRNAs act primarily on oncogenes and have achieved important clinical results, we believe that saRNAs targeting tumor suppressor genes may play an important role in the treatment of GC.

Materials And Methods

Collection of gastric cancer tissues and normal tissues

106 GC tissues and corresponding adjacent non-tumorous gastric samples were obtained from Shandong Provincial Hospital Affiliated to Shandong First Medical University between 2013 and 2018. No local or systemic treatment was conducted in these patients before the operation. The study was approved by the Research Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University. Informed consents were obtained from all patients.

Cell lines and culture

Authenticated GC cell lines (SGC-7901 and MKN-28) and the human gastric mucosal epithelial cell line (GES-1) were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin under a humidified atmosphere containing 5% CO_2 at 37°C.

Cell transfection

SGC-7901 and MKN-28 cells (2×10^5/6-well plate) were cultured to 50%–70% confluence. Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was used to transfect the cells with 50 nM saRNA targeting retinoblastoma (Rb) or negative control saRNA (dsControl). The mock control was treated with Lipofectamine 2000 alone.

RNA extraction and quantitative reverse-transcription PCR

Total RNA from tissues or cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse transcribed using the RT-PCR kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Quantitative PCR was conducted using the SYBR Green Premix reagent (Tiangen) and ABI Prism 7000 Fluorescent Quantitative PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The sequences of the primers used in this study are as follows: forward, 5'- CTC TCG TCA GGC TTG AGT TTG-3' and reverse, 5'- GAC ATC TCA TCT AGG TCA ACT GC -3' for Rb; and forward, 5'-GGA GCG AGA TCC CTC CAA AAT-3' and reverse, 5'-GGC TGT TGT CAT ACT TCT CAT GG-3' for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The PCR data were analyzed using the 2^{-DDCt} method, and expression was normalized to that of GAPDH.

Western blotting

Total cellular proteins were extracted using 1% NP-40 and 1 mM phenylmethylsulfonyl fluoride and were quantified using the bicinchoninic acid assay (Sigma-Aldrich, St. Louis, MO, USA). The proteins were then separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. After sequential incubations with primary and secondary antibodies, the bands were visualized by enhanced chemiluminescence using the Immobilon^{TM} Western Kit (Millipore, Burlington, MA, USA). GAPDH served as a loading control.

Cell proliferation assay

The proliferation of the GC cell lines SGC-7901 and MKN-28 was assessed using the Cell Counting Kit-8 (CCK8: Beyotime Biotechnology, Shanghai, China). All procedures were performed according to the manufacturer's instructions.

Cell migration and invasion assays

After 72 h, the cells were harvested following treatment with the Rb-targeting saRNA, dsControl, mock transfection, or saRNA/short-hairpin RNA combinations. Cell invasion and migration were measured via Transwell assays using chambers with membranes containing 8 μm pores (Corning, Corning, NY, USA). The cells were suspended in 100 μL serum-free medium and seeded in the upper chamber, and the lower chamber was filled with 500 μL complete media. After incubation at 37°C for 24 h, the Transwell membranes were fixed and stained with 0.5% Crystal Violet/methanol solution for 30 min. The cells that did not migrate were removed using cotton swabs, and the membranes were photographed using a digital camera. In the cell invasion assay, the Transwell membranes were coated with Matrigel (BD Biosciences, San Jose, CA, USA), and the cell incubation period was 48 h.

In vivo model of tumor growth

All animal experiments were approved by the Animal Care and Ethics Committee of Shandong Provincial Hospital affiliated to Shandong University and conducted in the Animal Center of Shandong Provincial Hospital affiliated to Shandong University. Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). The Rb-targeting saRNA and dsControl saRNA were transfected into GC cells, and the mock control group consisted of GC cells treated with the transfection reagent only. Nude mice were randomly divided into three groups (n = 6) consisting of mice injected with the following: (1) GC cells transfected with the Rb-targeting saRNA (saRNA group), (2) GC cells transfected with the dsControl saRNA (dsControl group), and (3) mock transfected GC cells (mock group). The cells (5×10^6) were subcutaneously injected into the right side of nude mice. After 21 days, the mice were sacrificed, and solid tumors were isolated for further analysis.

Statistical analysis

All experimental results are expressed as the mean ± standard deviation, and one-way analysis of variance followed by the Tukey–Kramer test for multiple comparisons were conducted using Prism statistical software (GraphPad, San Diego, CA, USA). Differences with a value of $P < 0.05$ were considered statistically significant.

Results

Rb was downregulated expressed in GC tissue and cells

In the first, we detected the expression of Rb in GC tissues and cells. As shown in Fig. 1A, Rb level was significantly decreased in GC tissues compared with the adjacent normal tissues. Besides, Rb expression in GC cells (MKN-28 and SGC-7901) was also significantly downregulated compared with the human gastric mucosal epithelial cell line (GES-1) (Fig. 1B).

saRNA transfection efficiency

First, we determined the transfection efficiency of the saRNAs targeting Rb. According to PCR, of the three saRNAs tested, saRNA-3 significantly increased the expression of Rb in both SGC-7901 and MKN-28 cells ($P < 0.01$). However, there was no significant difference in Rb expression between the control groups ($P > 0.05$) and the cells transfected with saRNA-1 or saRNA-2 (Fig. 2).

Significant activation of Rb expression by saRNA in GC cells

Next, our date showed that saRNA-3 notably increase the Rb expression levels in mRNA and protein (Fig.3). The Rb mRNA (4.96 folds or 4.53 folds) and protein (2.87 folds or 2.64 folds) levels after transfected with saRNA-3 were increased compared with the mock group ($P < 0.01$).

Effective inhibition of in vitro proliferation by saRNA in GC cells

The CCK-8 assay was used to detect the effect of saRNA-3 on SGC-7901 and MKN-28 cell proliferation. saRNA-3 significantly inhibited the proliferation of both cell types by day 3 after transfection. In addition, there was no significant difference in proliferation between the mock and dsControl groups (Fig. 4).

Effective inhibition of in vivo proliferation by saRNA in GC cells

For *in vivo* validation, we established a GC model by subcutaneously inoculating cells into BALB/c nude mice. saRNA-3 decreased the tumor volume and weight compared with the mock and dsControl groups, consistent with the *in vitro* results (Fig. 5).

Effective inhibition of migration and invasion by saRNA in GC cells

saRNA-3 transfection decreased the migratory ability of SGC-701 and MKN-28 cells compared with the control groups ($P < 0.01$; Fig. 6). Conversely, there was no significant difference between the dsControl and mock groups ($P > 0.05$). Moreover, the invasion assay showed that saRNA-3-transfected SGC-701

and MKN-28 cells exhibited a lower invasion rate compared with the dsControl and mock groups ($P < 0.01$; Fig. 7).

Discussion

Globally, GC is the third most lethal tumor, with a mortality rate of approximately 723,000 patients per year. Despite advances in medical technologies, the clinical manifestations of GC are not obvious, and it is usually not detected until an advanced stage. Therefore, the mortality of GC patients therefore has remained high. GC is a heterogeneous disease associated with a variety of genetic backgrounds and epigenetic changes, including genetic mutations, somatic cell copy number changes, structural variations, and epigenetic variations. Characterizing the molecular mechanisms involved in GC progression is therefore a difficult challenge. However, identifying new molecular targets in GC may lead to novel approaches to determine disease prognosis and suitable clinical treatment strategies.

Since the discovery of saRNAs, different RNAa models have been developed, and numerous studies have been conducted on this class of molecules. Janowski and Li et al. confirmed that different saRNAs activate E-cadherin, p21, VEGF, and PR and inhibit tumor cell growth [13, 14]. Junxia et al. reported that saRNAs inhibit breast and bladder cancers by activating E-cadherin [15]. Kang et al. established an orthotopic tumor model in mice and treated bladder cancer cells with relevant saRNA liquid particles to successfully inhibit tumor growth [16]. Ren also confirmed this mouse model using a similar experiment. Furthermore, saRNAs inhibited prostate cancer cell growth and prolonged survival in mice [17]. In our previous study, we designed an saRNA targeting the *VEZT* gene and found it inhibited the proliferation, invasion, and migration of GC cells via upregulated expression of VEZT [18].

Kundson et al. evaluated the genetic basis of childhood retinoblastoma in 1971 and found that tumor formation requires simultaneous deletion of 13q14 or inactivation of a pair of alleles of the Rb gene. Lee et al. sequenced and predicted that the protein corresponded to this RNA, and the gene was identified and isolated in 1989. Because the gene is closely related to the occurrence of retinoblastoma, it was named the *Rb* gene, which was the first successfully cloned tumor suppressor gene [19]. Recently, it was reported that the Rb gene is closely related to the development of many malignant tumors such as retinoblastomas, osteosarcomas [20], breast cancer [21], bladder cancer [22], and lung cancer [23]. Furthermore, inactivation of Rb contributes to tumor formation.

In the present study, we first used three saRNAs to reactivate Rb expression in GC cells (SGC-7901 and MKN-28) and selected saRNA-3 because it most effectively upregulated Rb expression. saRNA-3 inhibited GC cell proliferation both *in vivo* and *in vitro*.

Conclusion

Our findings also confirmed that saRNA-3 significantly inhibited GC cell invasion and migration. These results indicate that saRNA-mediated upregulation of Rb inhibited the tumorigenicity of GC cells.

Targeting specific genes via saRNAs may provide new therapeutic options that could significantly advance the treatment of cancer and other diseases. However, additional studies are needed to identify the mechanism of RNAa, the optimal method for designing saRNAs, and the impact of RNAa on other treatments.

Abbreviations

GC: Gastric cancer;

Rb: Retinoblastoma

saRNAs: Small activating RNAs

RNAa: RNA activation

dsControl: negative control saRNA

Declarations

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shandong Provincial Hospital affiliated to Shandong University. Written informed consent was obtained from all enrolled subjects.

Consent to publish

Not applicable.

Availability of data and materials

The data used to support the findings of this study are included in the article.

Competing Interest

There are no conflicts of interest to declare.

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Authors' contributions

WGC conceived the study. PLP, ZSJ and KS designed the experiments; TSB, XT and PLP conducted the experiments. PLP, WGC and ZSJ wrote the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

Figure.1

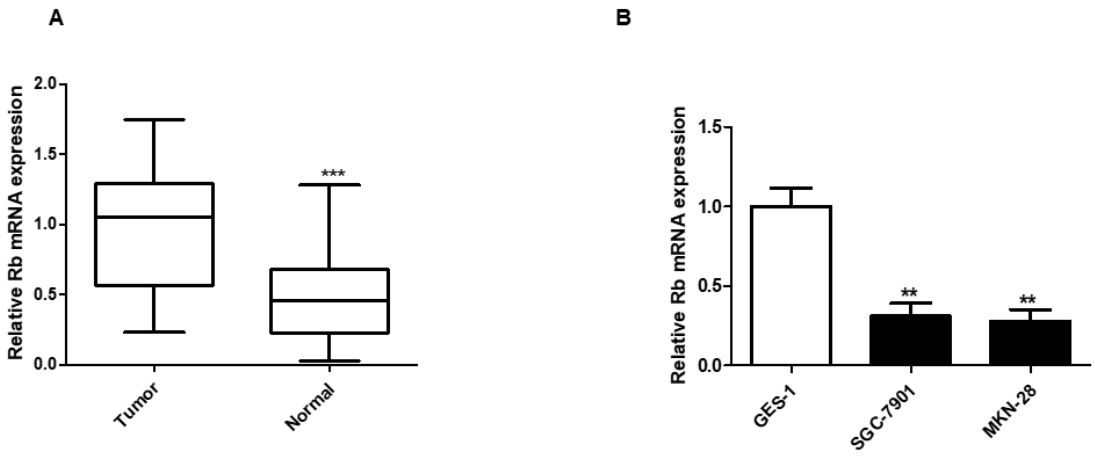

Figure 1

The retinoblastoma (Rb) was significantly downregulated in GC tissue and cells. (A) The expression of Rb was detected by qRT-PCR in GC tissues and adjacent normal tissues. (B) The expression of Rb in cells was detected using qRT-PCR. **P < 0.01, ***P < 0.001 compared with the Normal or GES-1 group.

Figure.2

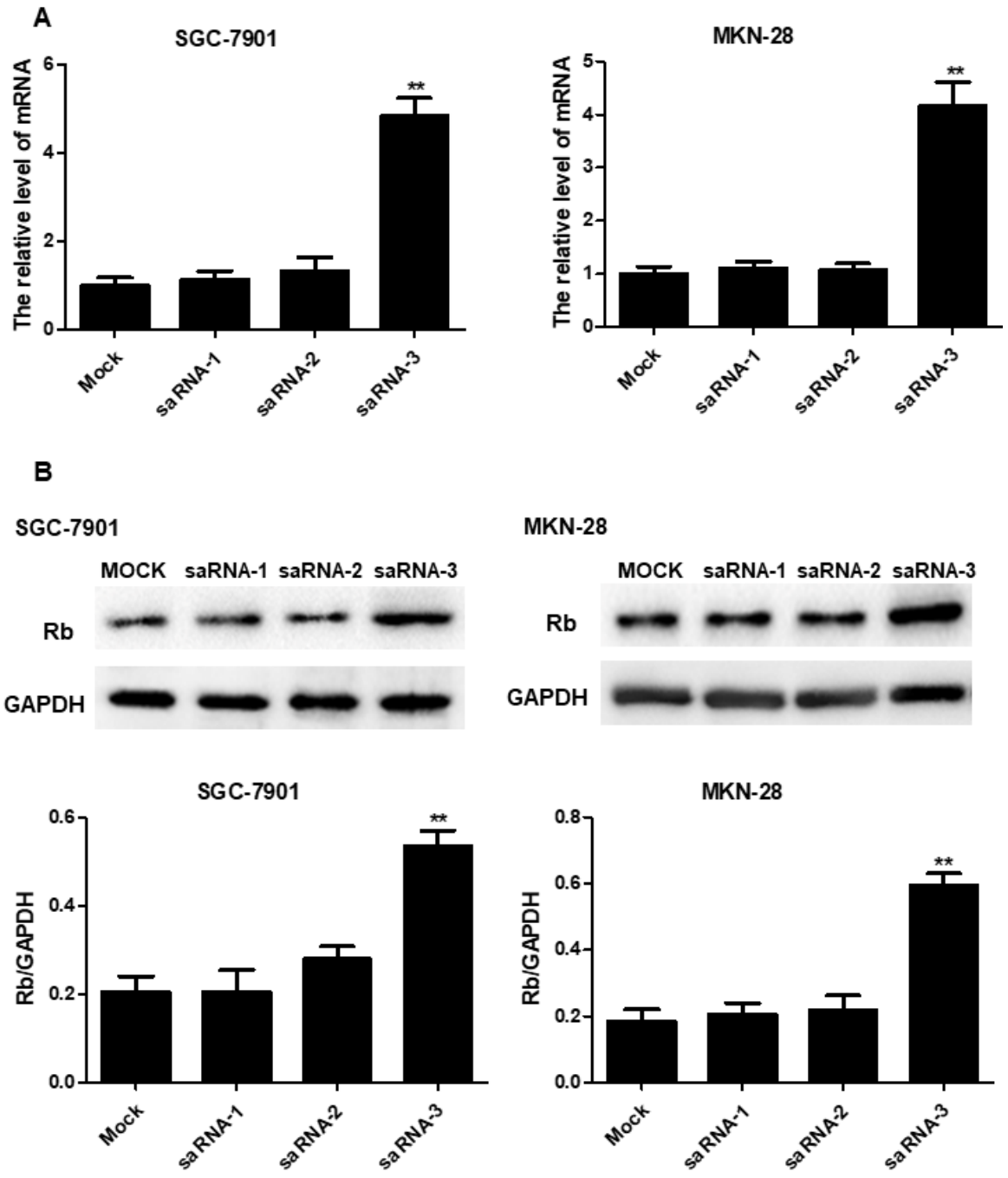

Figure 2

Different saRNAs reactivated the retinoblastoma (Rb) gene with different efficacies. SGC-7901 and MKN-28 cells were treated with 50 nM saRNA for 72 h. (A) The Rb mRNA level was assessed by real-time PCR. (B) The Rb protein level was assessed by western blotting. *P < 0.05, **P < 0.01 compared with the mock control.

Figure.3

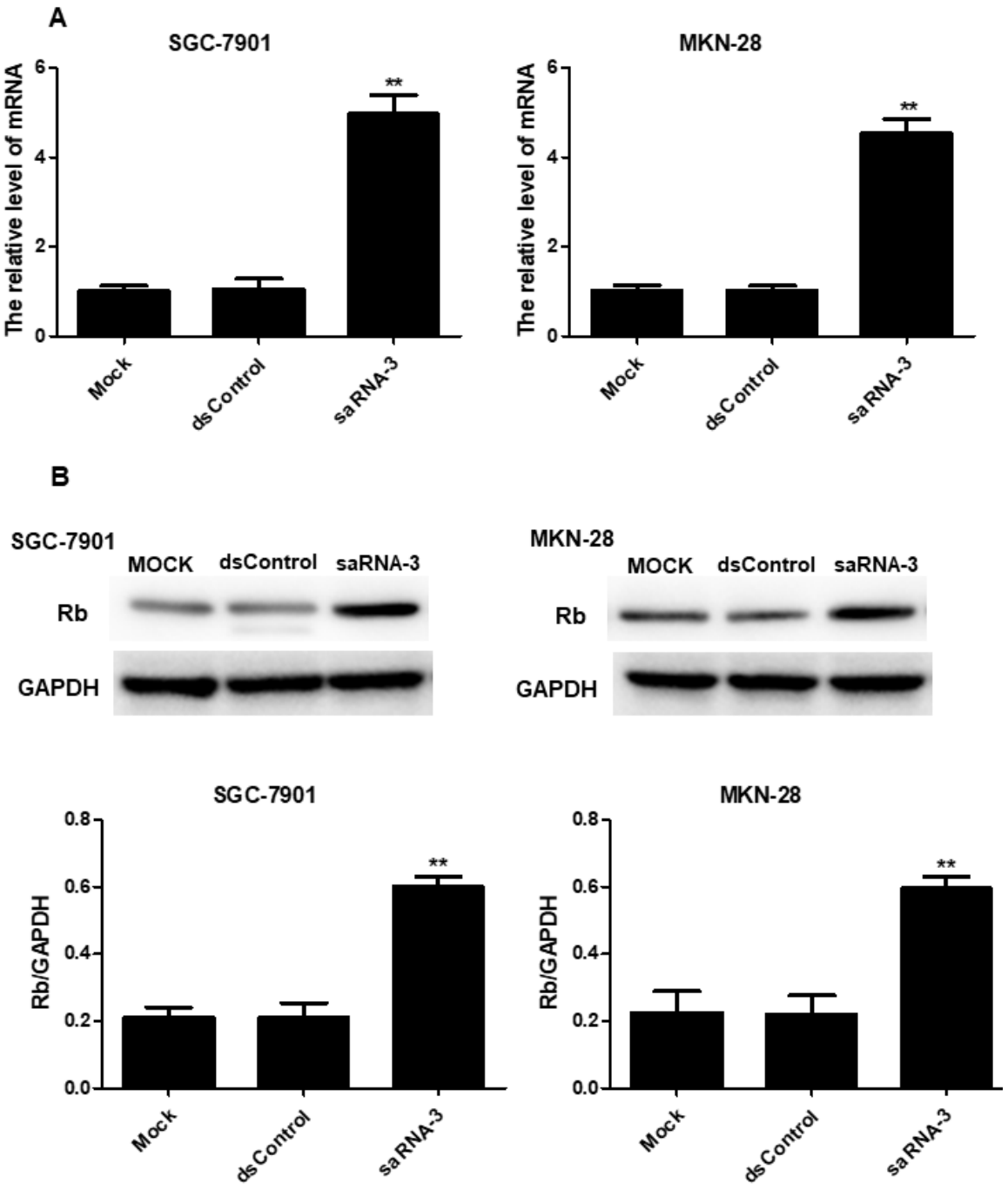

Figure 3

saRNA-3 induced upregulation of retinoblastoma (Rb) levels. SGC-7901 and MKN-28 cells were treated as described above. The levels of Rb mRNA (A) and protein (B) were detected by real-time PCR and western blot analysis, respectively. *P < 0.05, **P < 0.01 compared with the mock control.

Figure.4

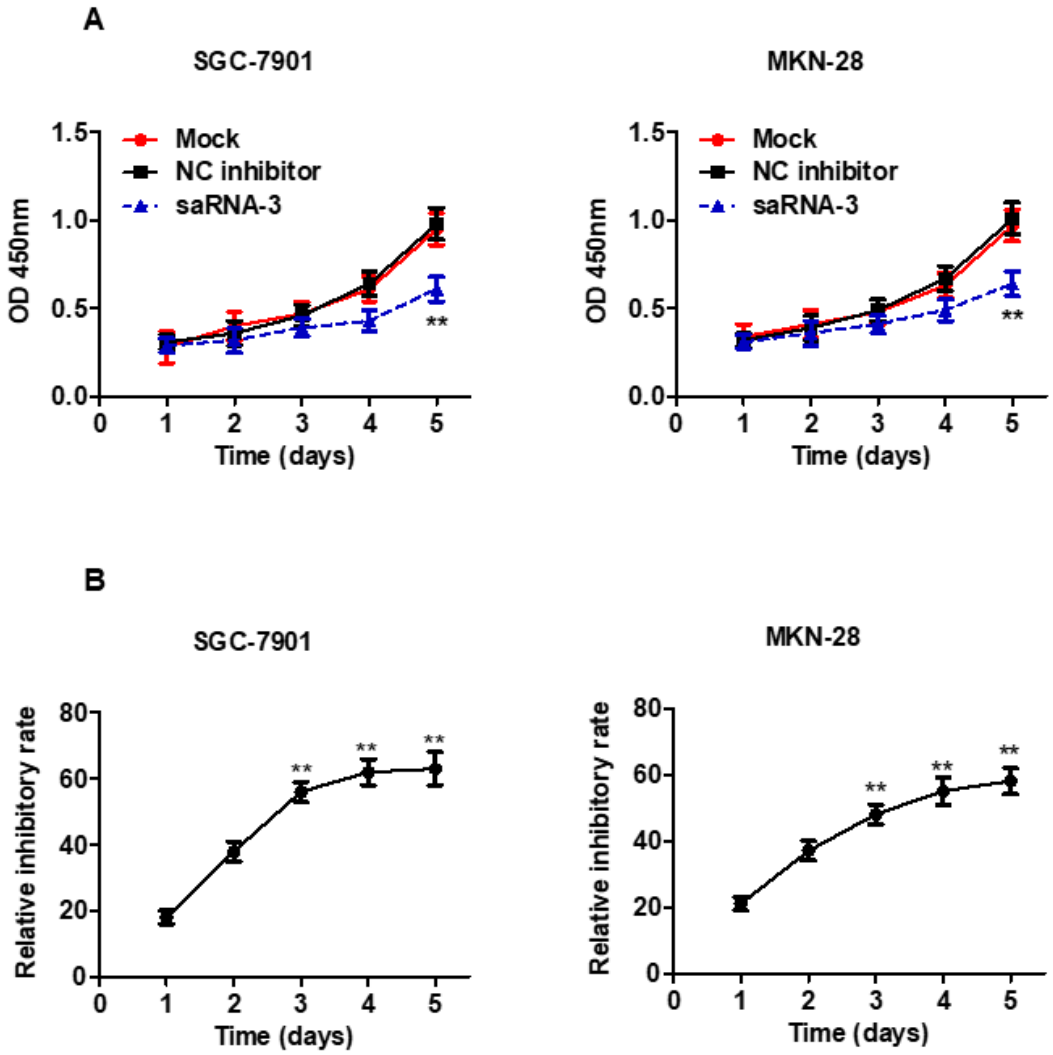

Figure 4

saRNA-3 inhibited the proliferation of gastric cancer cells in vitro. SGC-7901 and MKN-28 cells were treated as described above. Cell proliferation was detected using the CCK-8 assay. saRNA-3 significantly inhibited the proliferation of SGC-7901 and MKN-28 cells by day 3 of treatment (A). There was no significant difference between the two control groups (B). *P < 0.05, **P < 0.01 compared with the mock control.

Figure.5

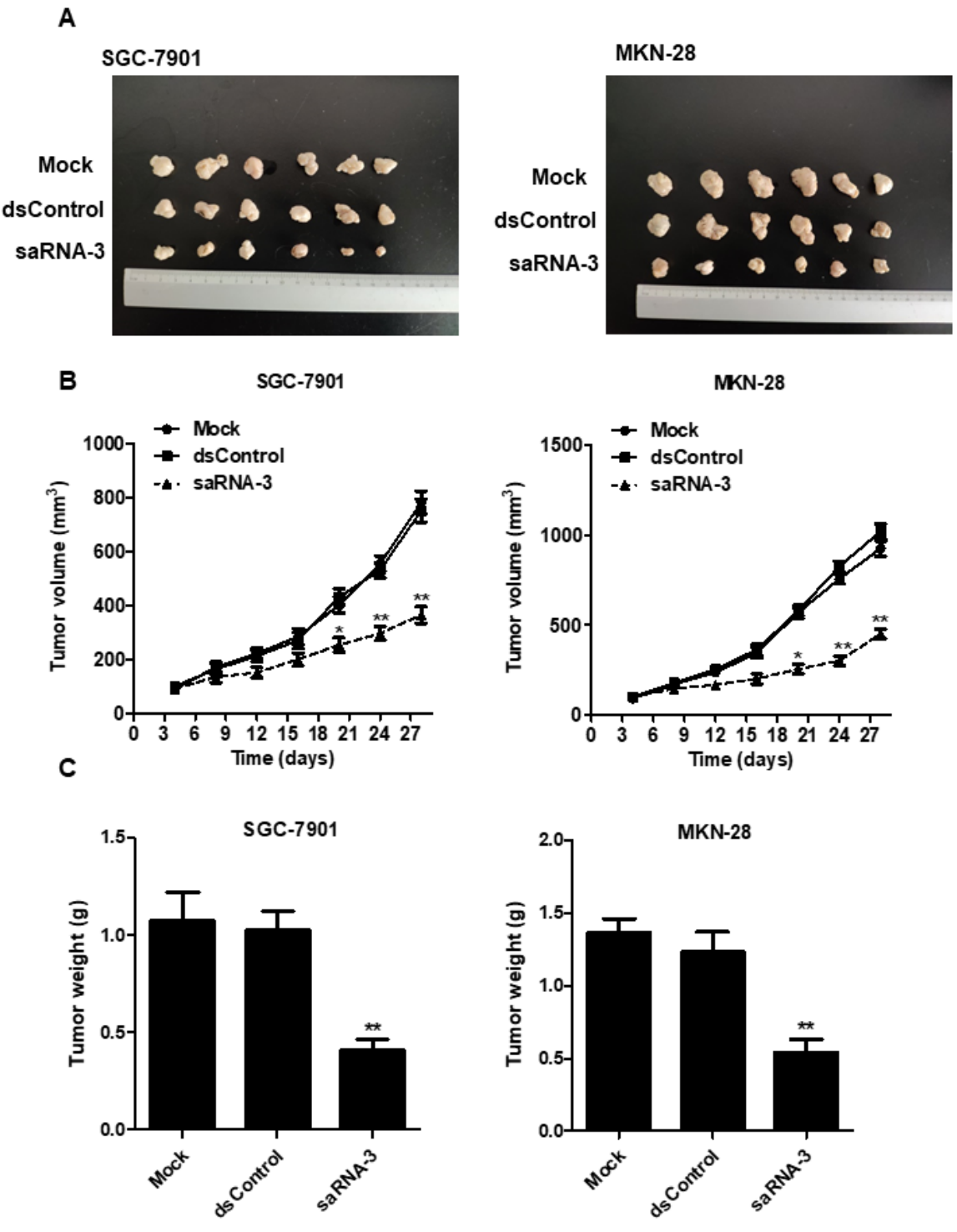

Figure 5

saRNA-3 suppressed tumor growth of gastric cancer cells in vivo. SGC-7901 and MKN-28 cells were treated as described above. Tumor tissue images (A), tumor growth curves (B), and the weight of tumor tissues at harvest (C). *P < 0.05, **P < 0.01 compared with the mock control.

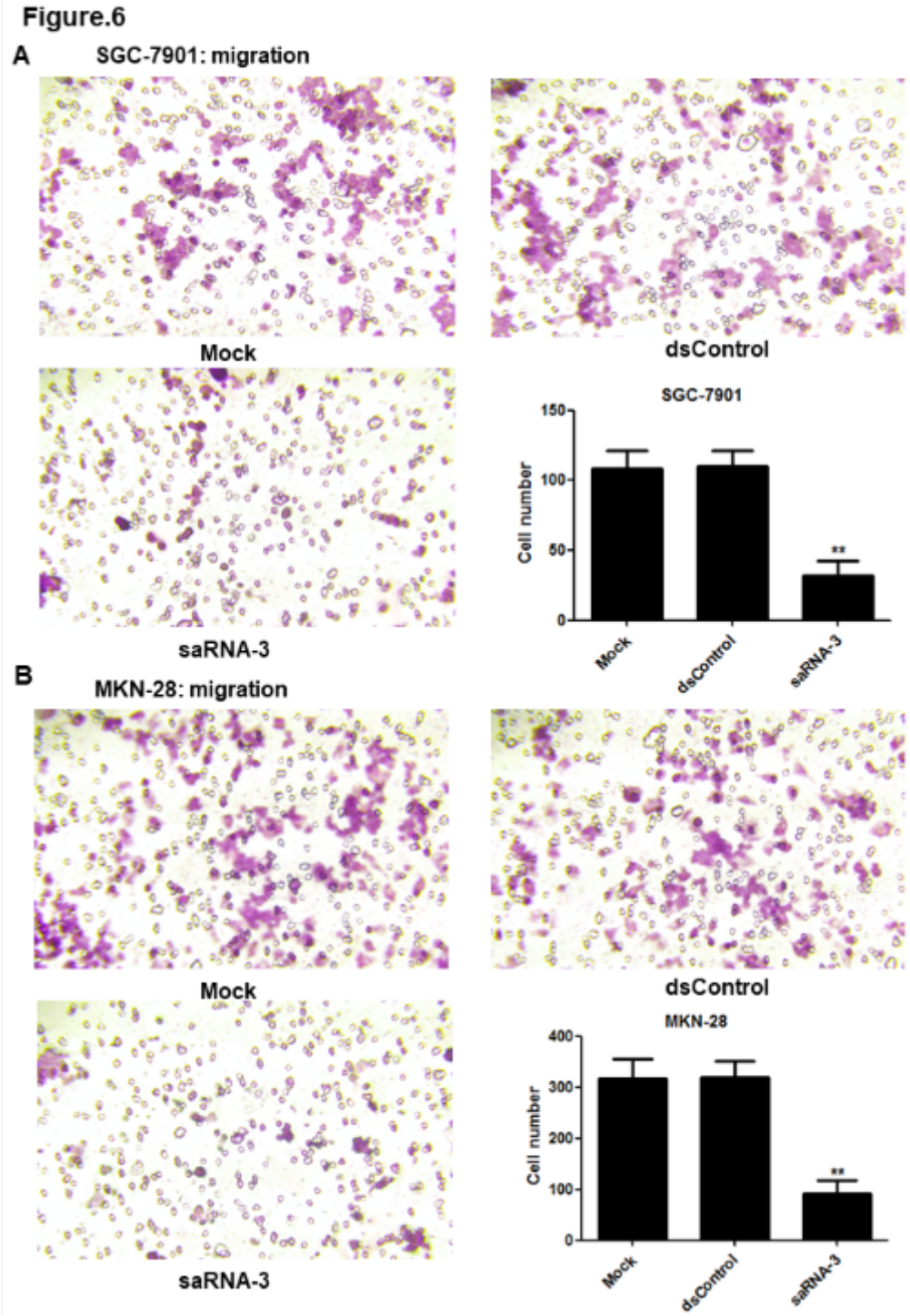

Figure 6

saRNA-3 inhibited the migration of gastric cancer cells. The numbers of migrating SGC-7901 (A) and MKN-28 (B) cells were significantly reduced after saRNA-3 transfection. *P < 0.05, **P < 0.01 compared with the mock control.

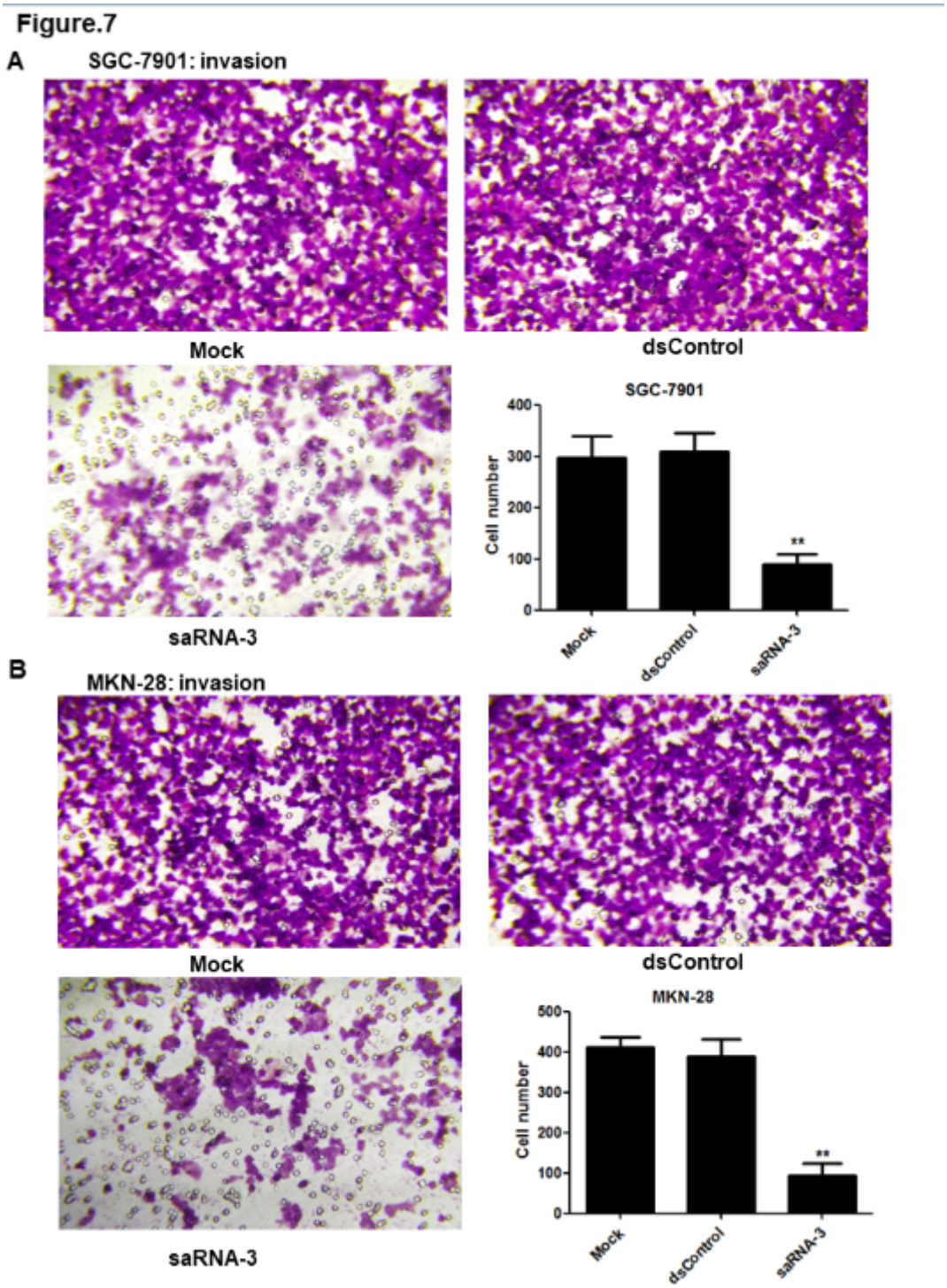

Figure 7

saRNA-3 inhibited the invasion of gastric cancer cells. The numbers of invading SGC-7901 (A) and MKN-28 (B) cells were significantly reduced after saRNA-3 transfection. *P < 0.05, **P < 0.01 compared with the mock control.