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MiR-199a-3p promotes gastric cancer progression by targeting ZHX1

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ABSTRACT

Accumulating evidence has indicated that microRNAs (miRNAs) act as critical epigenetic regulators in tumor carcinogenesis. Here, we report that miR-199a-3p was significantly upregulated in gastric cancer (GC) cell lines and tissues. Functional studies demonstrated that miR-199a-3p dramatically increased cell proliferation and suppressed cell apoptosis both in vitro and in vivo. Furthermore, the transcriptional regulator zinc fingers and homeoboxes 1 (*ZHX1*) was identified as one of the direct downstream targets of miR-199a-3p, miR-199a-3p bound to the *ZHX1* 3′ untranslated region (3′UTR) to regulate *ZHX1* protein expression. In addition, the expression of miR-199a-3p was inversely associated with that of *ZHX1* in GC cell lines. Overexpression of miR-199a-3p in SGC-7901 cells inhibited *ZHX1* expression, while reduction in miR-199a-3p by inhibitors in NCI-N87 cells enhanced *ZHX1* expression. Moreover, restoring *ZHX1* expression in SGC-7901/miR-199a-3p cells inhibited the cell proliferation induced by miR-199a-3p. Taken together, these findings suggest that miR-199a-3p may function as a novel tumor promoter in GC and its oncogenic activity may involve the direct targeting and inhibition of *ZHX1*.

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1. Introduction

Gastric cancer (GC) is one of the most common cancers and the second lethal cancer worldwide [1]. Although mortality rates of GC have decreased significantly, the absolute number of GC cases and deaths is still a big burden [2]. Therefore, a better comprehending of the molecular pathways related to the carcinogenesis of GC is essential to reduce GC mortality. Evidence collected to date has indicated that the microRNAs (miRNAs) are newfangled regulators of tumor development and new targets for cancer treatments in GC [3].

MiRNAs are small non-coding, single-stranded endogenous RNA molecules that degrade mRNAs and suppress protein expression [4]. Compelling evidences had proved that miRNAs modulate most cellular processes involved in tumor biological behaviors such as cell proliferation, apoptosis, differentiation and metastasis [5]. Abnormal expression of miRNAs, which function as oncogenes or tumor suppressor genes, can exist in many malignant tumors and is closely related to the occurrence and development of tumors

[6]. Although plenty of miRNAs in relation to GC have been studied, the underlying mechanism of these miRNAs in carcinogenesis of GC remains to be investigated.

MiR-199 was identified from mouse skin cells and it was cloned from the human osteoblast sarcoma cell [7]. The expression levels of miR-199a-3p vary in different tumor cells, and highly expressed miR-199a-3p can role as a potential oncogene in breast cancer [8], while suppressed miR-199a-3p can act as a possible tumor suppressor in hepatocellular carcinoma [9]. Our previous study found that the expression of miR-199a-3p in the plasma was substantially up-regulated in GC [10] and that miR-199a-3p in the plasma could serve as a potential diagnostic marker for early GC [11]. MiR-199a has been reported in recent studies on GC [12–14], while the role of miR-199a-3p in GC progression has not been explored.

In the present study, we demonstrated a general increase in the miR-199a-3p expression level in 52 GC tissues compared with the non-tumor tissues, and found that the miR-199a-3p expression levels are associated with tumor invasion and the TNM stage. In addition, we discovered that miR-199-3p could induce the growth and inhibit apoptosis of GC cells both in vitro and in vivo by directly targeting the transcription factor zinc fingers and homeobox 1 (*ZHX1*).

2. Materials and methods

2.1. Cell culture and clinical specimens

Preparation of clinical samples, gastric cancer cell lines AGS, MKN-45, MKN-28, SGC-7901, NCI-N87, BGC-823, immortalized normal gastric mucosal epithelial cell line (GES-1) and human embryonic kidney cell line 293T (HEK 293T) was described in [15]. The six gastric cell lines and GES-1 cells were grown in RPMI-1640, while HEK 293T was grown in DMEM (Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cell lines and clinical specimens using an mirVana™ miRNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The expression level of miR-199a-3p in cell lines and tissues was measured by qRT-PCR according to the Taqman® MicroRNA Assays protocol (Applied Biosystems) and normalized using U6 small nuclear RNA (RNU6B; Applied Biosystems) by the $2^{-\Delta CT}$ method. The relative expression ratio of miR-199a-3p in each paired tumor and non-tumor tissue was calculated by the $2^{-\Delta\Delta CT}$ method. The expression level of ZHX1 mRNA was measured by qRT-PCR according to the SYBR Green real-time PCR Assay protocol (Applied Biosystems). The GAPDH mRNA level was used for normalization. The expression level of ZHX1 mRNA relative to GAPDH mRNA was calculated using the $2^{-\Delta CT}$ method.

2.3. Transient transfection

MiR-199a-3p mimics (dsRNA oligonucleotides), negative control (NC), miR-199a-3p inhibitor, and inhibitor negative control were purchased from GenePharma (Shanghai, China). The pEGFP-ZHX1 (1–873) plasmid was donated by Prof. Kazuya Yamada (Matsumoto University Graduate School of Health Science). Transfection of cells with oligonucleotides was performed using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) at a final concentration of 100 nM. Transfection efficiency was monitored by qRT-PCR.

2.4. Cell proliferation assay

At 24 h post transfection with miR-199a-3p mimics/inhibitors or control oligonucleotides, cells were seeded into 96-well plates (1.5×10^5 cells/well) and cell proliferation was documented every 24 h for 4 days. Cell proliferation was assessed in triplicates by the water-soluble tetrazolium salt (WST) assay using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) following the manufacturer's instructions.

2.5. Soft agar colony formation assay

MiRNA mimics/inhibitors-transfected SGC-7901/NCI-N87 cells were resuspended with 0.3% soft agar (A9045, low gelling temperature, Sigma–Aldrich, St. Louis, MO, USA) in RPMI 1640 containing 10% FBS and layered on 0.6% solidified agar in RPMI 1640 containing 10% FBS in six-well plates (1×10^3 cells/well) 24 h post transfection. The plates were incubated for 2 weeks. Colonies containing at least 50 cells were counted.

2.6. Flow cytometric analysis for apoptosis

One day before transfection, 2×10^6 cells were seeded into six-well culture plates without antibiotics. The cells were transfected with miR-199a-3p mimics/miR-199a-3p inhibitor or their respective controls. Forty-eight hours after transfection, cells were harvested and stained with the AnnexinV/PI double staining kit (BD Biosciences, USA) according to the manufacturer's protocol. Apoptotic cells were assessed in triplicates by flow cytometry on a FACScan (Beckman Instruments, Fullerton, CA, USA). Experiments were repeated three times.

2.7. Lentiviral transfection for stable expression clones

LV3-pGLV-H1-GFP+Puro plasmids with hsa-miR-199a-3p inhibitor or control oligonucleotides, namely LV-anti-miR-199a-3p and LV-anti-miR-NC, were purchased from GenePharma (Shanghai, China). Lentivirus transfections were performed according to the manufacturer's instructions to establish anti-miR-199a-3p-expressing stable clones in NCI-87 cells (NCI-N87/anti-miR). The control clones (NCI-N87/anti-NC) were produced by a similar method. The miR-199a-3p expression level was examined by qRT-PCR using U6 RNA as an endogenous control.

2.8. Tumour xenograft model, tumorigenicity assay and immunohistochemistry

All animal studies complied with protocols approved by the Committee on Animal Care in Shanghai Jiao Tong University School of Medicine. Cells (100 ml, 2×10^6 cells) from stable transfected lines LV-anti-miR-199a-3p and LV-anti-miR-NC were collected and inoculated subcutaneously into the right flank of 4-week-old male nude mice. Five mice were used for each group. Mice were checked weekly, and tumor nodules were measured with a caliper. After the mice were sacrificed, all tumor grafts were excised, weighed, harvested, fixed, embedded and examined histologically.

2.9. Cloning of 3′UTR of ZHX1 into pMIR-REPORT luciferase vector

The 3′UTRs of ZHX1 was synthesized according to a ∼59-bp genomic fragment containing the predicted miR-199a-3p binding sites. The mutant ZHX1 3′UTR construct was designed to mutate three intermittent nucleotides complementary to the miR-199a-3p seed-region. Both strands were annealed and cloned into the Hind III-MluI sites of the pMIR-REPORT miRNA expression reporter vector (Applied Biosystems).

2.10. Western blot analysis

ZHX1 protein levels were quantified by standard immunoblot procedures, using rabbit anti-human ZHX1 antibody (1:2000, Abcam, USA). Monoclonal anti-GAPDH (1:5000, Abcam, USA) was used for loading control.

2.11. Statistical analysis

Student's t test or one-way ANOVA were used for statistical analysis when appropriate. All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). A two-tailed value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. The expression of miR-199a-3p is up-regulated in GC and correlates with TNM stage and tumor invasion

MiR-199a-3p expression was examined by quantitative real time RT-PCR (qRT-PCR) in GC cell lines and GES-1. As shown in Fig. 1 A, miR-199a-3p was generally upregulated in all GC cell lines

compared with GES-1. The expression of miR-199a-3p was further evaluated by qRT-PCR in tumor samples and their matched non-tumor samples from 52 GC patients (Fig. 1B). The average expression level of miR-199a-3p was significantly upregulated in tumor samples compared with the non-tumor samples (Fig. 1C). These results strongly indicated that miR-199a-3p was significantly upregulated in GC cells and tissues.

The correlations between expression level of miR-199a-3p and clinicopathologic factors in GC were examined. Clinicopathologic characteristics of 52 GC patients are shown in Table 1. Among the 52 GC tissues, 69.2% (36/52) of the tumor tissues exhibited upregulation of miR-199a-3p (relative expression ratio < 1.0). In addition, 57.6% (30/52) of GC samples showed increased expression of miR-199a-3p under the twofold cut-off (relative expression ratio < 0.5). 52 cases were divided into two groups: miR-199a-3p

low expression ($n = 22$) and miR-199a-3p high expression ($n = 30$). The miR-199a-3p high-expression group had inclinations toward the TNM stage ($P = 0.037$) and invasion depth ($P = 0.08$). However, there were not any relationships between the miR-199a-3p expression and other clinicopathological factors.

3.2. Ectopic expression of miR-199a-3p induces GC cell proliferation

Considering that miR-199a-3p is significantly upregulated in GC, it may role as a tumor promoter. Therefore, to further investigate the inducing function of miR-199a-3p in GC, SGC-7901 cells, which presented a low level of miR-199a-3p, were transfected with miR-199a-3p mimics or negative control (NC). In addition, NCI-N87 cells, which expressed a high level of miR-199a-3p, were transfected with miR-199a-3p inhibitors or its control. The WST cell growth assay was performed on the transfected cell lines. As indicated in Fig. 2A and B (3.4 ± 0.2 VS 2.7 ± 0.4, $P < 0.05$; 1.5 ± 0.4 VS 1.0 ± 0.1, $P < 0.01$), SCG-7901 cells transfected with miR-199a-3p mimics showed marked cell growth induction while NCI-N87 cells transfected with inhibitors showed significant cell growth inhibition. To further identify the effect of miR-199a-3p on cell growth, the soft agar colony formation assay on transfected cells was performed. The number of colonies of SGC-7901 cells transfected with miR-199a-3p mimics was higher than half that of the control ($161\% \pm 25.2\%$ VS $51.7\% \pm 27.9\%$, $P < 0.01$, Fig. 2C). Opposite results were observed with NCI-N87 cells transfected with inhibitors ($126.8\% \pm 9.42\%$ VS $163.5\% \pm 14.6\%$, $P < 0.01$, Fig. 2D). These results exhibited that miR-199a-3p can promote the tumorigenicity of GC cells in vitro.

3.3. Downregulation of miR-199a-3p expression promotes GC cells apoptosis

GC cell apoptosis analysis was carried out to elucidate the mechanism of miR-199a-3p-mediated cell growth promotion in GC cells. The results showed that comparing with the control group, SGC-7901 cells transfected with miR-199a-3p mimics
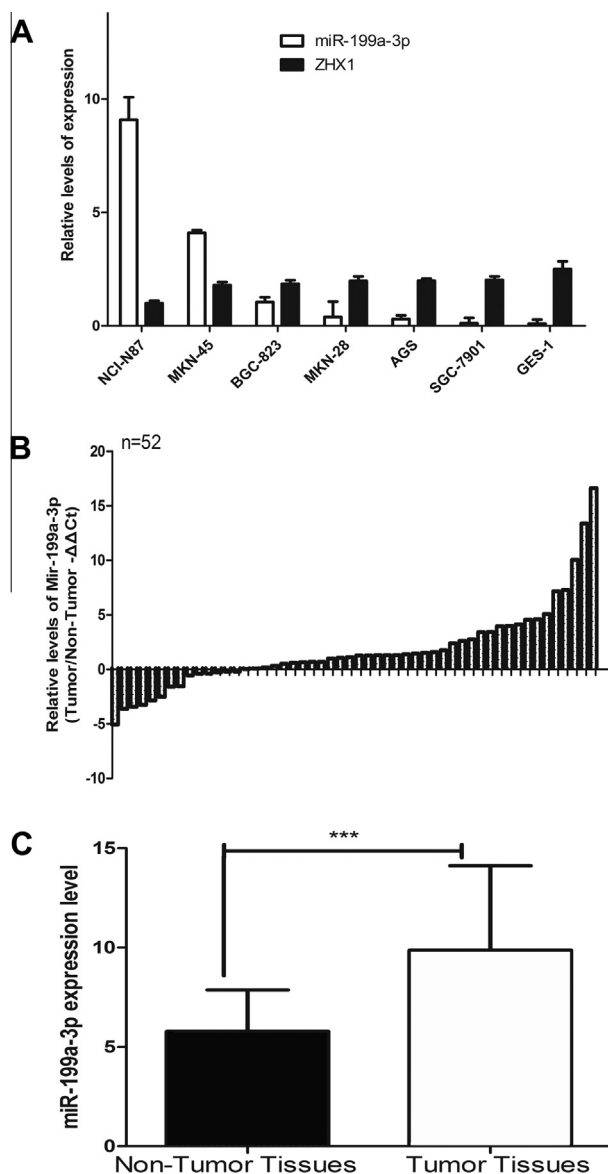

Fig. 1. Upregulation of miR-199a-3p expression in GC cell lines and tissues compared with the corresponding controls. (A) Relative expression of miR-199a-3p and ZHX1 in six GC cell lines and GES-1 was carried out by qRT-PCR. (B) Relative expression of miR-199a-3p in 52 GC patient tissues compared with its adjacent non-tumor tissues. QRT-PCR results are shown $-\triangle\triangle$CT values. (C) The mean and standard deviation of miR-199a-3p expression levels in 52 surgical specimens of GC tissues and matched non-tumor tissues were shown. Data are presented $2^{(-\triangle CT)}$ values (***$P < 0.001$).

Table 1
Relationship between miR-199a-3p expression level and clinicopathologic parameters in 52 GC cases.

Clinicopathologic parameters	MiR-199a-3p expression		P-value
	Low ($N = 22$)	High ($N = 30$)	
Ages (years)			
⩾60	16	19	0.390
<60	6	11	
Gender			
Female	6	12	0.390
Male	16	18	
Tumor size (cm)			
⩾5	10	9	0.382
<5	12	21	
Differentiation			
Well, moderately	13	9	0.054
Poorly, undifferentiated	9	21	
Location			
Distal third	17	25	0.752
Middle third, proximal third	5	5	
Tumor invasion			
T1, T2	8	3	0.037[*]
T3, T4	14	27	
Lymph node metastasis			
Yes	12	26	0.058
No	10	4	
TNM stage			
I,II	10	3	0.008[*]
III,IV	12	27	

[*] P-value < 0.05 was considered statistically significant.
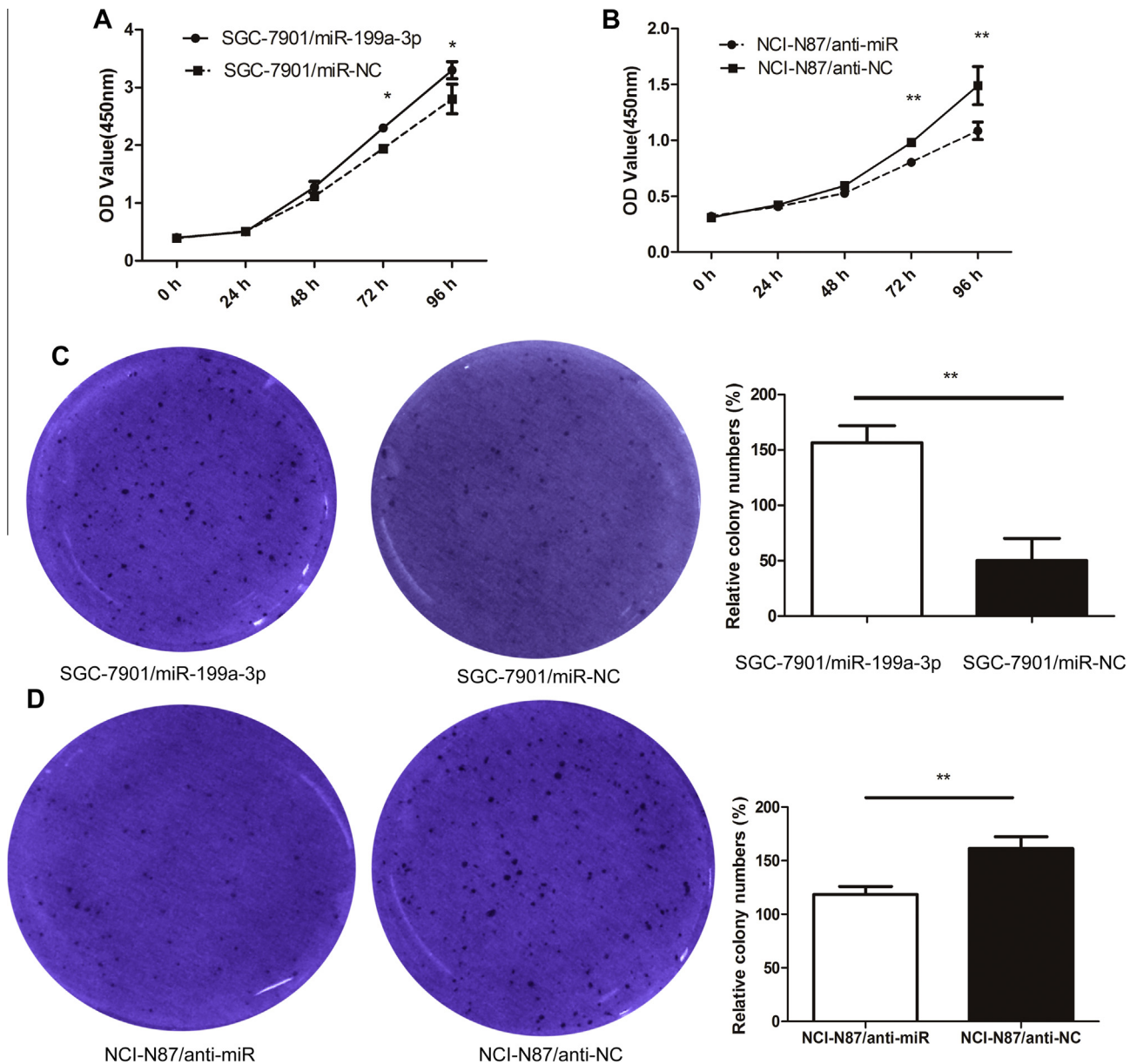
Fig. 2. Up/down expression of miR-199a-3p promotes/inhibits the proliferation of GC cells in vitro. Cell proliferation was evaluated by the WST assay in SGC-7901 cells (A) and NCI-N87 cells (B). SGC-7901 Cells were transfected with miR-199a-3p mimics or NC and NCI-N87 transfected with anti-miR-199a-3p or anti-miR-NC at 24 h post-transfection were subjected to WST assay. The results are means of three independent experiments ± S.D. ($^{*}P < 0.05$, $^{**}P < 0.01$). The effect of miR-199a-3p and anti-miR-199a-3p on cell proliferation was assessed by the colony formation assay in SGC-7901 cells (C) and NCI-N87 cells (D). SGC-7901 Cells transfected with 100 nM miR-199a-3p mimics or control and NCI-N87 transfected inhibitors or control were seeded onto 6-well plates. The number of colonies was counted on the 14th day after seeding. Representative photographs of colonies are shown in left panels. Relevant quantification of the results is shown in the bar graphs. The results are means of three independent experiments ± S.D. ($^{**}P < 0.01$).

showed an obvious decrease in the apoptotic rate (13.4% ± 2.1% VS 21.3% ± 3.1%, $P < 0.05$, Fig. 3A). On the contrary, the apoptotic rate of miR-199a-3p inhibitor-transfected NCI-N87 cells was obviously higher compared with the negative control (28.9% ± 1.2% VS 18.7% ± 3.4%, $P < 0.01$, Fig. 3B). However, there were no significant differences in the cell cycle between the different groups (data not shown). These results suggest that overexpression of miR-199a-3p inhibited apoptosis in GC cells, which may contribute to the growth induction features of miR-199a-3p.

Cysteine-aspartic protease (caspase) 3 is responsible for regulating and initiating apoptosis of mature cells, and the importance of caspase-3 in GC has been confirmed in many studies [16]. Thus, we investigated the expression of caspase-3 to demonstrate the influence of miR-199a-3p on GC cell apoptosis by regulating caspase-3. The expression of caspase-3 did not show a significant difference among the different cell groups, while cleaved caspase-3 showed remarkable differences. As shown in Fig. 4, the expression of cleaved caspase-3 was significantly lower in SGC-7901 cells transfected with the miR-199a-3p mimics compared with that in SGC-7901 cells transfected with the negative control. NCI-N87 cells transfected with the miR-199a-3p inhibitors showed higher expression of cleaved caspase-3 than NCI-N87 cells transfected with the negative control.

3.4. MiR-199a-3p Directly targets ZHX1 3′UTR to inhibit ZHX1 expression

To investigate new targets of miR-199a-3p and better knowing the mechanisms underlying the tumor suppressive effect of downregulation of miR-199a-3p in GC, the bioinformatics tools,
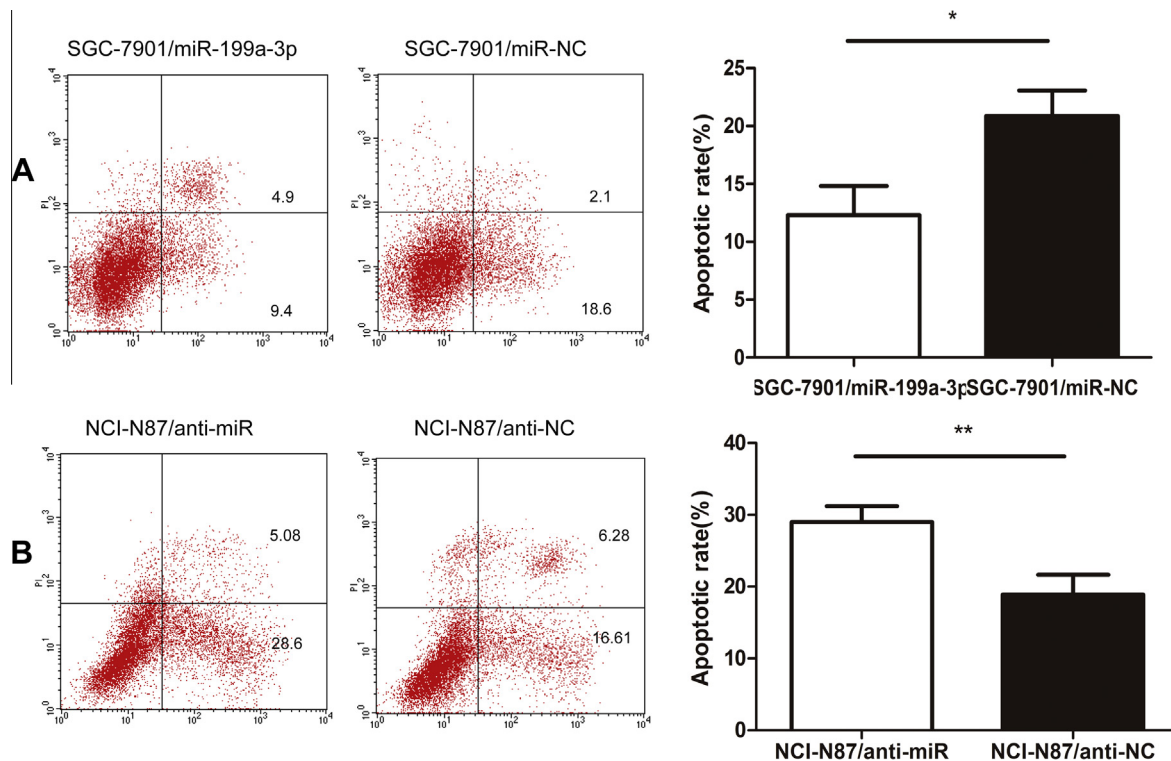
Fig. 3. Effect of miR-199a-3p on apoptosis in GC cells. (A) Representative histograms depicting apoptosis of SGC-7901 cells transiently transfected with 100 nM miR-199a-3p mimics or NC and parental cells (left panels). The percentage of apoptotic cells of three independent experiments ± S.D. are shown in the bar graphs ($^*P < 0.05$). (B) Representative histograms depicting apoptosis of NCI-N87 cells transiently transfected with 100 nM miR-199a-3p inhibitors or NC and parental cells (left panels). The percentage of apoptotic cells of three independent experiments ± S.D. are shown in the bar graphs ($^{**}P < 0.01$).

TargetScan, RNAhybrid and miRanda algorithms, were used for target search. Among numerous predicted candidate genes, the transcription repressor *ZHX1* is of special interest and showed the highest score. Importantly, *ZHX1* play a suppressive role in cell growth in HCC [9], but the function of *ZHX1* in GC has not been reported yet.

Luciferase reporter assays were carried out to identify a direct interaction between miR-199a-3p and the 3′UTR of *ZHX1*. Wide-type and mutated luciferase reporters were constructed (Fig. 4A). The pMIR/*ZHX1*/wt and pMIR/*ZHX1*/mut luciferase reporter constructs were transfected into HEK 293T cells, along with miR-199a-3p or NC mimics. The relative luciferase activity of the pMIR/*ZHX1*/wt reporter was obviously inhibited (1.49 ± 0.41 VS 0.81 ± 0.24, $P < 0.05$; 0.75 ± 0.18 VS 0.79 ± 0.31, $P > 0.05$, Fig. 4B). This result indicated that the 3′UTR of *ZHX1* contains direct binding sites for miR-199a-3p. To determine whether miR-199a-3p down-regulated *ZHX1* at the mRNA and/or protein level, we examined *ZHX1* expression by qRT-PCR and Western blotting. As shown in Fig. 4C higher panel, the *ZHX1* protein level was lower in miR-199a-3p mimics-transfected SGC-7901 cells compared with that in the NC mimics-transfected cells. In Fig. 4C lower panel, ZHX1 protein level was higher in pEGFP-ZHX1-transfected SGC-7901cells, which means up-regulate ZHX1 successfully by transient transfection. However, miR-199a-3p did have no impact on *ZHX1* mRNA level (Fig. 4D). These results suggested that miR-199a-3p negatively regulates *ZHX1* expression at the translational level.

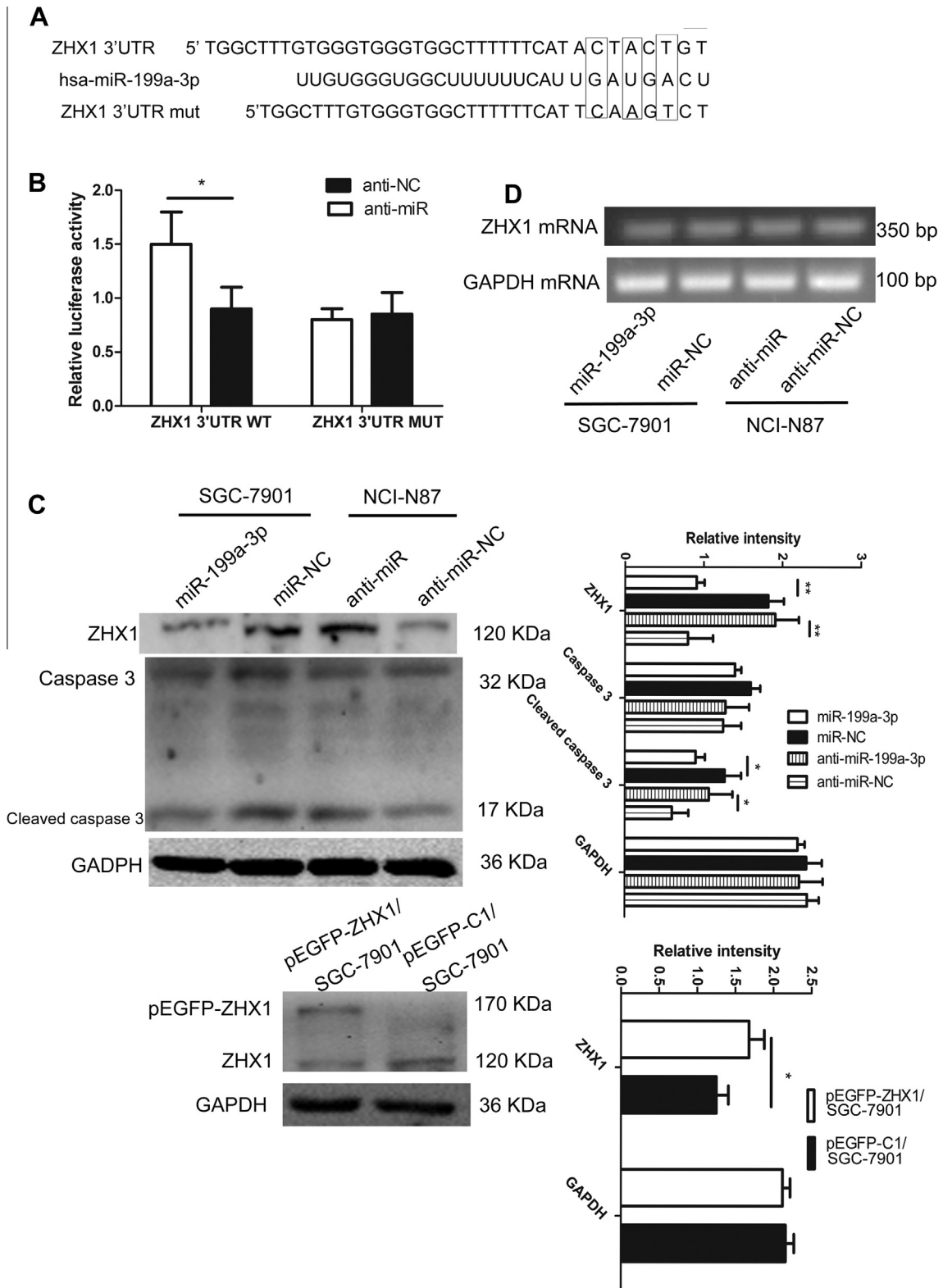### 3.5. Downexpression of miR-199a-3p suppresses tumorigenicity and promotes apoptosis in vivo

Given that downregulation of miR-199a-3p inhibits GC cell proliferation and induces apoptosis in vitro, we identify whether suppression of miR-199a-3p could suppress tumor growth and induce apoptosis in vivo. NCI-N87 cells with LV-anti-miR-199a-3p (NCI-N87/anti-miR) or LV-anti-miR-control (NCI-N87/anti-NC) were acquired as indicated in the Materials and methods. Cells were injected subcutaneously into nude mice. The tumor latency time was significantly different between the mice injected with NCI-N87/anti-miR cells and those injected with NCI-N87/anti-NC cells. Tumors derived from the NCI-N87/anti-miR cells grew substantially slower than those derived from the NCI-N87/anti-NC cells throughout the tumor growth (1.41 ± 0.15 VS 0.68 ± 0.17, $P < 0.01$, Fig. 5A and B). The average weight of tumors resulting from NCI-N87/anti-miR cells was obviously lower than that of tumors derived from NCI-N87/anti-NC cells (1.31 ± 0.52 VS 0.56 ± 0.12, $P < 0.05$, Fig. 5C and D).

The impact of miR-199a-3p on cell proliferation in tumors was evaluated by examining the proliferative index of Ki-67. Immunohistochemistry staining suggested that the Ki-67 staining was significantly lower in the NCI-N87/anti-miR tumors compared with that in the NCI-N87/miR-NC tumors. Additionally, TUNEL assays on tumor tissues were examined. As shown in Fig. 5E and F($71.3\% \pm 12.1\%$ VS $21\% \pm 9.62\%$, $P < 0.01$; $26.1\% \pm 8.67\%$ VS $119.2\% \pm 28.3\%$, $P < 0.01$), NCI-87/anti-miR cells showed a less tumor cell positive staining and a significantly higher apoptosis index than the NCI-N87/anti-NC. These results indicated that decreasing expression of miR-199a-3p contributed to inhibition of tumorigenicity and promotion of apoptosis in vivo.

3.6. Overexpression of ZHX1 rescues effect of miR-199a-3p in GC cells

Because miR-199a-3p suppressed *ZHX1* to induce cell proliferation and inhibit apoptosis, overexpression of *ZHX1* could reverse this phenomenon. Indeed, when the *ZHX1* expression plasmid (pEGFP-*ZHX1*) was imported into SGC-7901 cells transiently trans-

A

ZHX1 3'UTR   5' TGG C TTTGTGGGTGGGTGGCTTTTTTCAT A C T A C T G T

hsa-miR-199a-3p   UUGUGGGUGGCUUUUUUCAU U G A U G A C U

ZHX1 3'UTR mut   5'TGGCTTTGTGGGTGGCTTTTTTCAT T C A A G T C T

Fig. 4. MiR-199a-3p targets ZHX1 and inhibits caspase 3 expression. (A) Sequence of the ZHX1 3'UTR showing the miR-199a-3p binding site and mutation of the ZHX1 3'UTR binding site to create *ZHX1*-mut. (B) miR-199a-3p inhibitors up-regulated luciferase activities controlled by wild-type *ZHX1* 3'UTR (*$P < 0.01$), but did not affect luciferase activity controlled by mutant *ZHX1* 3'UTR. The results are means of three independent experiments ± S.D. (C) Representative Western blotting images of indicated protein in SGC-7901 cells and NCI-N87 (left panels), with relevant quantification (right panel). The results are means of three independent experiments ± S.D. (*$P < 0.01$, **$P < 0.01$) *ZHX1* protein was detected in SGC-7901 transfected with pEGFP-*ZHX1* and pEGFP-C1 (lower panel). The results are means of three independent experiments ± S.D. (*$P < 0.01$) (D) *ZHX1* mRNA in SGC-7901 cells and NCI-N87 were analyzed by RT-PCR at 48 h post-transfection with miR-199a-3p mimics or NC and miR-199a-3p inhibitors or NC.

fected with miR-199a-3p mimics, the inducing function of miR-199a-3p on cell growth was partially reversed, assessed by the WST cell growth assay ($3.1 ± 0.2$ VS $2.41 ± 0.34$, $2.39 ± 2.19$,

Fig. 6A). Moreover, apoptosis rates were amplified when *ZHX1* was overexpressed in SGC-7901 cells transfected with miR-199a-3p mimics ($14.3\% ± 1.2\%$, $7.2\% ± 1.31\%$, $21.7\% ± 2.6\%$, Fig. 6B,C).
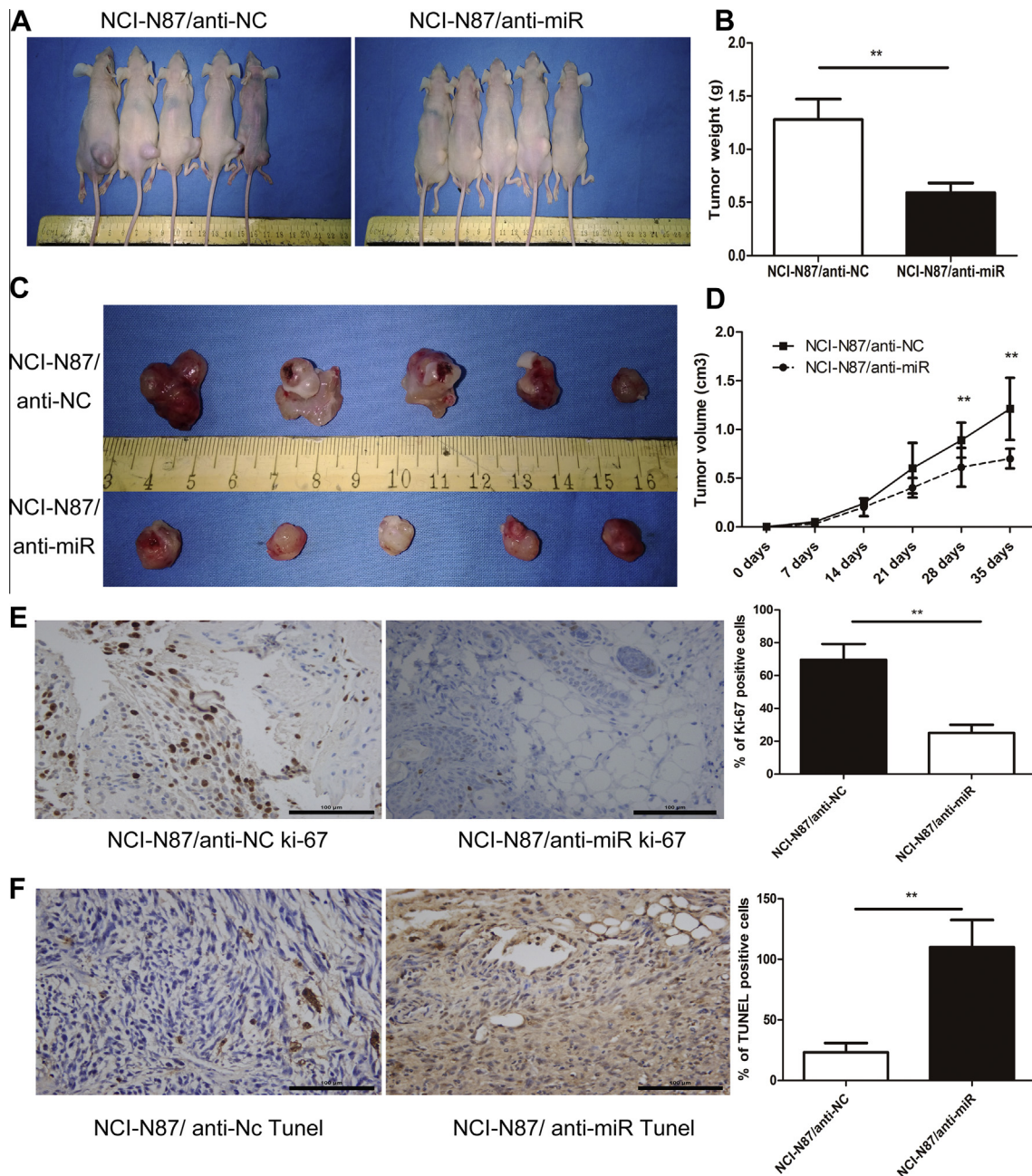
Fig. 5. Underexpression of miR-199a-3p inhibits tumorigenicity and increases apoptosis in vivo. (A and C) Photographs of tumors derived from NCI-N87/anti-NC and NCI-N87/anti-miR in nude mice. (B) Average weight of tumors in nude mice. Means ± S.D. was shown ($^{**}P < 0.01$). (D) Growth kinetics of tumors in nude mice. Tumor diameters were measured every 7 days. Bars ± S.D. ($^{**}P < 0.01$). (E–F) Representative photographs of immunohistochemical analysis of Ki-67 antigen in tumors of nude mice. Representative image of TUNEL assay of tumor specimens from nude mice (original magnification, 200×). Comparison of proliferation index. Means ± S.D. are shown ($^{**}P < 0.01$). The percentage of apoptotic cells was counted. Columns, mean; bars, ±S.D. ($^{**}P < 0.01$).

These data indicated that growth promotion and apoptosis inhibition by miR-199a-3p are related to *ZHX1* expression.

4. Discussion

MiRNAs have an significant role in regulating cellular activities. They can profoundly affect the expression of a large number of genes that encode proteins. Expression level of miR-199a-3p varies among related cancers, resulting in different effects, e.g., miR-199a-3p acts as a protective factor in HCC [9] while it can induce cell proliferation in breast cancer [8]. MiR-199a had been reported to be upregulated in GC tissues [12–14,17]. Previously, we demonstrated that miR-199a-3p was upregulated in GC patients' plasma compared with normal people, and that plasma miR-199a-3p may be a novel diagnostic biomarker for GC detection, even at early stages [10,11]. Hence, we were interested in investigating the mechanism by which miR-199a-3p affects the carcinogenesis of GC.

In this study, we found that miR-199a-3p expressed much higher in 69.2% of the GC samples, indicating that increased miR-199a-3p expression is common in GC. Importantly, miR-199a-3p levels positively associated with the TNM stage and the deeper tumor invasion of GC patients. The functional studies suggested that the expression of miR-199a-3p inhibitors in NCI-N87 cells resulted in a significant inhibitory effect on cell proliferation, whereas a more aggressive phenotype in vitro resulted from
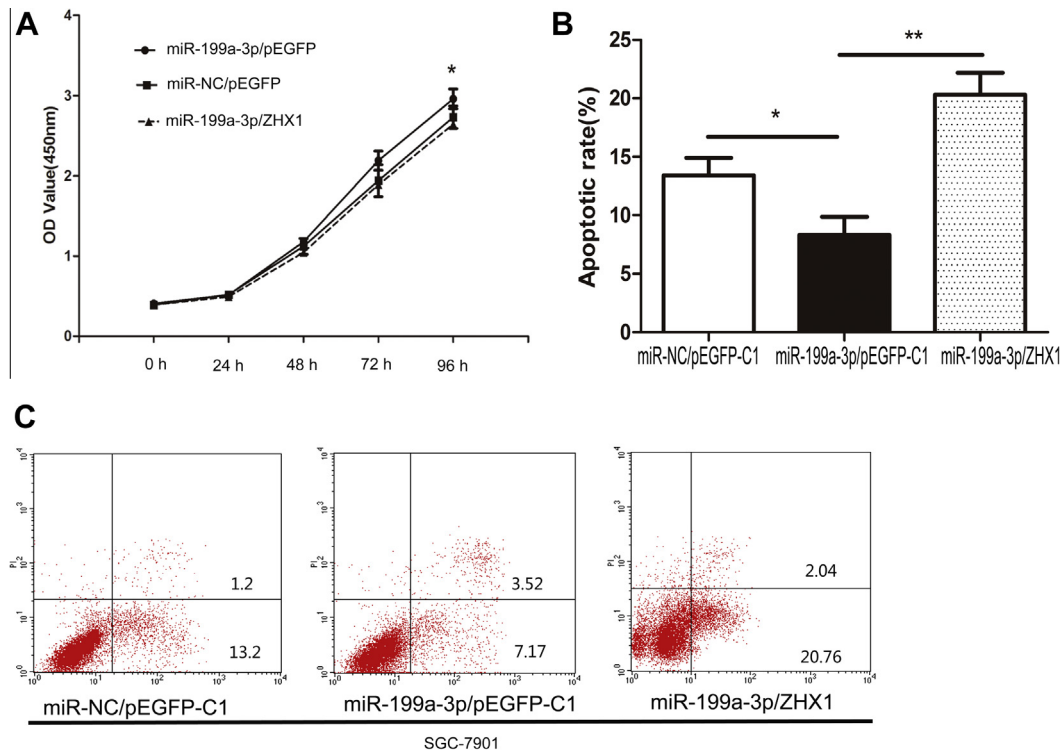
Fig. 6. Overexpression of *ZHX1* rescues effect of miR-199a-3p in GC cells. Cells were transfected with miR-199a-3p mimics or control, together with *ZHX1* expression vector (pEGFP-*ZHX1*) or pEGFP-C1 at 24 h post-transfection were subjected to WST assay. Upon transfected with pEGFP-*ZHX1*, SGC-7901 (A) were partially rescued from miR-199a-3p growth induction properties. Representative histograms depicting apoptosis SGC-7901 cells (B and C) transfected with miR-199a-3p mimics or control, together with pEGFP-*ZHX1* or pEGFP-C1 by flow cytometry. The percentage of apoptotic cells of three independent experiments (*$P < 0.01$, **$P < 0.01$) are shown in the bar graphs.

overexpression of miR-199a-3p in SGC-7901 cells. Furthermore, in vivo stable low expression of miR-199a-3p in NCI-N87 cells showed a weaker ability to induce tumor growth compared with the same cell lines transfected with the negative control vectors. These results, together with other studies [18], suggested that miR-199a-3p is a tumor-inducing miRNA in GC.

To better knowing the functional mechanism of miR-199a-3p as a tumor-inducing miRNA, we used bioinformatics analyses to identify the genes downstream of miR-199a-3p. Smad4, klotho and MAP3K11 had been proven to be direct targets of miR-199a-3p [12–14]. Here, we demonstrated that *ZHX1* is a brand new downstream target of miR-199a-3p, and that *ZHX1* expression was regulated by miR-199a-3p via direct binding to the 3′UTR of *ZHX1*. This was supported by the fact that miR-199a-3p expression inhibited the luciferase activity and *ZHX1* protein expression, whereas anti-miR-199a-3p expression promoted the *ZHX1* protein expression. However, miR-199a-3p did not affect the *ZHX1* mRNA level. MiRNAs can modulate gene activity via decreasing gene translation or increasing degradation of target mRNA [19]. *ZHX1* belongs to the zinc-fingers class of homeodomain transcription factors, and its function remains unclear [20,21] The *ZHX1* gene has not been thoroughly investigated in the area of cancer. In HCC, Wang et al. [22] have found that overexpression of *ZHX1* in SMMC-7721 cells inhibited proliferation, while reduced *ZHX1* expression is widespread among cancer tissues from HCC patients, and a low expression of *ZHX1* may be responsible for hepatocarcinogenesis.

Caspases are a set of cysteine-containing proteolytic enzymes produced, which are associated with proliferation, cell migration and organization. They execute apoptosis, and caspase-3 seems to be the best-known member of the caspase family. The importance of this caspase in GC has been confirmed in numerous studies and a great deal of research continues to be carried out. In the present study, Western blot analysis demonstrated that upregulated miR-199a-3p in SGC-7901 cells suppressed the level of

cleaved caspase-3 protein compared with that in the negative control. Furthermore, downregulation of miR-199a-3p in NCI-N87 cells led to a higher expression level of cleaved caspase-3 than that in the negative control. The change in cleaved caspase-3, the active form of caspase-3, was inversely associated with the expression of miR-199a-3p, which indicated that the growth of GC cells induced by miR-199a-3p may partially be related to the suppression of caspase-3 and its active form's expression. At the same time, we observed a corresponding change in cleaved caspase-3 after elevating *ZHX1* expression in NCI-N87 cells. Importantly, overexpression of *ZHX1* rescued the miR-199a-3p-induced cell growth and cell apoptosis inhibition, further suggesting that *ZHX1* is a direct target of miR-199a-3p, indicating an essential role for *ZHX1* as a mediator of the biological function of miR-199a-3p in GC.

In conclusion, we found that miR-199a-3p plays a tumor-inducing role in GC cells. The inducing function of miR-199a-3p was exerted by downregulating the protein expression of its direct target, *ZHX1*. Although miR-199a-3p's function in cancer is not entirely clear, our findings provided a novel molecular target and mechanism of miR-199a-3p in GC. Therefore, miR-199a-3p could be considered as a novel target for GC prevention and therapy.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.09.047.

References

[1] A. Jemal et al., Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 2010 19 1893–1907.

[2] Jemal, A. et al. (2010) Global patterns of cancer incidence and mortality rates and trends. Cancer Epidemiol. Biomark. Prev. 19, 1893–1907.

[3] Cho, W. (2010) MicroRNAs in cancer—from research to therapy. Biochim. Biophys. Acta (BBA) – Rev. Cancer 1805, 209–217.

[4] Bartel, D.P. (2009) MicroRNAs: target recognition and regulatory functions. Cell 136, 215–233.

[5] Nicoloso, M.S. et al. (2009) MicroRNAs—the micro steering wheel of tumour metastases. Nat. Rev. Cancer 9, 293–302.

[6] Alvarez-Garcia, I. et al. (2005) MicroRNA functions in animal development and human disease. Development 132, 4653–4662.

[7] Lagos-Quintana, M. et al. (2003) New microRNAs from mouse and human. RNA 9, 175–179.

[8] Shatseva, T. et al. (2011) MicroRNA miR-199a-3p regulates cell proliferation and survival by targeting caveolin-2. J. Cell Sci. 124, 2826–2836.

[9] Murakami, Y. et al. (2006) Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene 25, 2537–2545.

[10] Li, C. et al. (2013) MiRNA-199a-3p in plasma as a potential diagnostic biomarker for gastric cancer. Ann. Surg. Oncol. 20 (Suppl. 3), S397–S405.

[11] Li, C. et al. (2013) MiRNA-199a-3p: a potential circulating diagnostic biomarker for early gastric cancer. J. Surg. Oncol. 108, 89–92.

[12] Song, G. et al. (2010) MiR-199a regulates the tumor suppressor mitogen-activated protein kinase kinase kinase 11 in gastric cancer. Biol. Pharmaceut. Bull. 12, 331822–331827.

[13] He, X.J. et al. (2014) Up-regulated miR-199a-5p in gastric cancer functions as an oncogene and targets klotho. BMC cancer 14, 218.

[14] Zhang, Y. et al. (2012) Functional screening for miRNAs targeting Smad4 identified miR-199a as a negative regulator of TGF-beta signalling pathway. Nucleic Acids Res. 40, 9286–9297.

[15] Feng, R. et al. (2010) MiR-126 functions as a tumour suppressor in human gastric cancer. Cancer Lett. 298, 50–63.

[16] Frejlich, E. et al. (2012) Caspases and their role in gastric cancer. Adv. Clin. Exp. Med. 22, 593–602.

[17] Ueda, T. et al. (2010) Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. Lancet Oncol. 11, 136–146.

[18] Brenner, B. et al. (2011) MicroRNAs as a potential prognostic factor in gastric cancer. World J. Gastroenterol. 17, 3976–3985.

[19] Breving, K. et al. (2010) The complexities of microRNA regulation: mirandering around the rules. Int. J. Biochem. Cell Biol. 42, 1316–1329.

[20] Chen, S. et al. (2013) The SUMOylation of zinc-fingers and homeoboxes 1 (ZHX1) by Ubc9 regulates its stability and transcriptional repression activity. J. Cell. Biochem. 114, 2323–2333.

[21] Yamada, K. et al. (2002) Functional analysis and the molecular dissection of zinc-fingers and homeoboxes 1 (ZHX1). Biochem. Biophys. Res. Commun. 297, 368–374.

[22] Wang, J. et al. (2013) Construction of a recombinant eukaryotic human ZHX1 gene expression plasmid and the role of ZHX1 in hepatocellular carcinoma. Mol. Med. Rep. 8, 1531–1536.