



# Circular RNA 0081146 facilitates the progression of gastric cancer by sponging miR-144 and up-regulating HMGB1

Qihua Xu · Bingling Liao · Sheng Hu · Ying Zhou · Wei Xia

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## Abstract

**Objectives** Recent studies have revealed that circular RNA (circRNA) plays a pivotal role in cancer development. The study aimed to investigate the role of circ\_0081146 in gastric cancer (GC).

**Results** Circ\_0081146 was upregulated in GC tissues and cells. Patients with high expression of circ\_0081146 had a significantly reduced 5-year overall survival rate. Circ\_0081146 knockdown restrained the growth, migration and invasion of GC cells in vitro as well as tumorigenesis in vivo. Circ\_0081146 targeted miR-144 and HMGB1 was

targeted by miR-144. Circ\_0081146 was negatively correlated with miR-144 expression, while positively correlated with HMGB1 expression in GC tissues. Moreover, the inhibitory effect of circ\_0081146 knockdown on the progression of GC cells were reversed by silencing miR-144 or HMGB1 overexpression. Mechanically, circ\_0081146 increased HMGB1 expression by targeting miR-144.

**Conclusion** Circ\_0081146 functions as an oncogene in GC to promote cell growth, migration and invasion via modulating the miR-144/HMGB1 axis.

**Keywords** Gastric cancer · Circ\_0081146 · MiR-144 · HMGB1

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## Introduction

Gastric cancer (GC) is the most common malignancy of the gastrointestinal tract (Karimi et al. 2014). In recent years, the incidence of GC has been gradually decreasing due to improvements in human nutrition and eradication of helicobacter pylori (Torre et al. 2015). However, the recurrence rate is increased due to distant metastasis of the disease and the 5-year mortality rate of GC patients is more than 70% (Zhang et al. 2017). The occurrence and metastasis of GC are regulated by many genes, so finding new molecular

mechanisms to activate or inactivate GC would be helpful for the early diagnosis of GC.

Circular RNAs (circRNAs) are endogenous non-coding RNAs (ncRNAs) that composed of closed covalent loops with the covalent union at the 3' and 5' end (Liu et al. 2018). There are three types of circRNAs containing exons, introns, and intergenomic regions classified through their origins (Ledford 2013), among them, exon circRNAs are the final products, which generally exist in the cytoplasm and are the most widely studied circRNAs (Vicens and Westhof 2014). Recently, accumulating evidence have confirmed that circRNAs played an important regulatory role in human cancers (Li et al. 2015), especially in GC. For instance, Huang et al. reported that hsa\_circ\_0008035 promoted GC tumorigenesis by modulating the proliferation and invasion of GC cells (Huang et al. 2019a). CircRNA AKT3 could enhance cisplatin resistance in GC (Huang et al. 2019b). Besides, circPVT1 was identified as a proliferating factor and prognostic marker in GC (Chen et al. 2017). Not long ago, Yuan et al. revealed that hsa-circ\_0081146 was enormously up-regulated in GC tissues by a multiple of 4 through microarray data analysis (Dang et al. 2017). However, the function of circ\_0081146 in GC is unknown.

MicroRNAs (miRNAs) have been shown to be small ncRNAs that play a central role in various biological processes (Ambros 2001), and they have thousands of target mRNAs and can regulate the expression of these target mRNAs (Ke et al. 2003). Accumulating evidence suggested that miRNAs were involved in the progression of various cancers, including GC (D'Angelo 2016; Miao et al. 2019). For example, miR-539 might be a prognosis biomarker of GC (Jin et al. 2019). Moreover, miR-181a functioned as a regulator of TGF- $\beta$  signaling to promote cell growth and metastasis in GC (Ge et al. 2019). MicroRNA-144 (MiR-144) was confirmed to be decreased in GC tissues and serum samples, suggesting that miR-144 might serve as a prognostic biomarker in GC (Liu et al. 2017). However, the role and underlying mechanism of miR-144 are still largely unknown.

High Mobility Group Box 1 (HMGB1) is often thought to be associated with inflammation-related cancers (Zhang et al. 2018), and it has an extracellular activity similar to cytokines, which can regulate inflammation, proliferation and migration in many

cancers (Yang et al. 2005). Also, HMGB1 could facilitate the development of many cancers (Li et al. 2019; Lv et al. 2019), including GC. Others like Suren et al. indicated that HMGB1 was associated with tumor grade, T stage and pTNM stage of gastric adenocarcinoma (Suren et al. 2018). Zhang et al. demonstrated that HMGB1 knockdown impaired growth and invasion of GC cells by modulating the NF- $\kappa$ B signal pathway in vitro and in vivo (Zhang et al. 2014). However, it remains unclear whether HMGB1 could be regulated by circRNAs in GC.

In our preliminary study, we checked the level of circ\_0081146 in GC tissues, serum and cells and identified circ\_0081146. In addition, we explored the effect of circ\_0081146 on GC progression by silencing circ\_0081146 and found the axis of miR-144/HMGB1 was modulated by circ\_0081146. Our study aimed to provide novel potential biomarkers for the diagnosis of GC.

## Materials and methods

### GC tissue samples and cell culture

GC tissues and paracancerous tissues were obtained from 50 GC patients at Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine, and frozen stored at  $-80^{\circ}\text{C}$ . Blood samples of 30 GC patients and 22 health controls (with no diagnosed cancer) were collected. All patients had written informed consents and no patients received any treatment before surgery. This study was permitted by the human ethics committee of Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine.

In this paper, human GC cell lines AGS, MGC-803 and HGC-27 were purchased from Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and gastric mucosal epithelial cells GES-1 were acquired from Procell (Wuhan, China). These cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640, Hyclone, South Logan, UT, USA) medium containing 10% fetal bovine serum (FBS, Hyclone) and 1 mg/mL penicillin/streptomycin (Solarbio, Beijing, China) in 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

## Transfection

Human lentivirus-sh-circ\_0081146 and the negative control sh-NC were synthesized by Wuyuan Company (Beijing, China). MiR-144 mimics (miR-144), mimics-NC (miR-NC), miR-144 inhibitor (anti-miR-144) and the overexpression plasmid of HMGB1 were bought from GenePharma (Shanghai, China). Both AGS and MGC-803 cells were transfected by using Lipofectamine 2000 (Solarbio) at a density of 60–70%.

RNase R treatment and quantitative real-time polymerase chain reaction (qRT-PCR).

For qRT-PCR detection, total RNA from GC tissues and cells was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) with RNeasy Mini Kit (QIAGEN, Shanghai, China). After RNase R treatment, complementary DNA (cDNA) was synthesized by Prime Script RT Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and cDNA amplification was performed on AB7300 thermo-recycler (Applied Biosystems, Foster City, CA, USA) using SYBR Select Master Mix (Applied Biosystems). In addition, the nuclear and cytoplasmic fractions were separated through a NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was applied as the standard internal control of circ\_0081146 and HMGB1, and U6 acted as an internal reference for miR-144. The relative level was analyzed by the  $2^{-\Delta\Delta C_t}$  method. The primer sequences in this paper were as follows: circ\_0081146, Forward (F): 5'-TGGCAGCCAGGGGGAAC-3', Reverse (R): 5'-TCCAGAAGGACCTCGGCTTC-3'. GAPDH, F: 5'-GGGAAGCTCACTGGCATGGCCTTCC-3', R: 5'-CATGTGGGCCATGAGGTCCACCAC-3'. U6, F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'. MiR-144, F: 5'-ATC-CAGTGCCTGTCGTG-3', R: 5'-TGCTTATACAGTATAGATG-3'. HMGB1, F: 5'-AAAGCGGACAAGGCCCGTTAT-3', R: 5'-AAGAGGAAGAAGGCCGAAGGAG-3'.

## Cell viability and proliferation assays

For viability detection of AGS and MGC-803 cells, these cells were uniformly added into 96-well plates. At different time points (0 h, 24 h, 48 h and 72 h) after transfection, 10  $\mu$ L of Cell Counting Kit-8 (CCK-

8, Dojindo Molecular Technologies, Tokyo, Japan) was added to well and further incubated for 3 h. Subsequently, the optical density (OD) at 450 nm was measured with a microplate reader (BioTek, Beijing, China).

Colony formation assay was used to detect proliferation of AGS and MGC-803 cells. The stably transfected cells of AGS and MGC-803 were tiled into 6-well plates and incubated for 2 weeks. The medium was replaced every 4 d. Then, the colonies were fixed and stained with 0.1% crystal violet (Solarbio) for 20 min. Finally, the visible colonies were counted. All experiments were performed with three replicates.

## Transwell assay

Transwell assay was used to examine cell migration (without Matrigel) and invasion (coated with Matrigel, Invitrogen). The starved AGS and MGC-803 cells in 100  $\mu$ L of serum-free medium were evenly added into the upper compartment of the chamber. 600  $\mu$ L of complete medium was used as a source of chemoattractant and was added to the bottom chamber. After 24-h incubation, cells migrated or invaded the bottom surface of the chamber were fixed with methanol and stained with 0.1% crystal violet (Solarbio) for 20 min. Then, the number of cells was counted by an inverted microscope.

## Western blot assay

Proteins in GC tissues and cells were dissolved using RIPA buffer (Sigma-Aldrich, St Louis, MO, USA). These proteins were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime, Shanghai, China) and transferred into polyvinylidene difluoride (PVDF) membranes (Beyotime). Next, the membranes were immersed in 5% skim milk powder for 2 h at 37 °C, and incubated with the primary antibodies against Proliferating Cell Nuclear Antigen (PCNA, 1:500, Thermo Fisher Scientific), Matrix Metalloprotein9 (MMP9, 1:2000, Thermo Fisher Scientific), HMGB1 (1:3000, Abcam, Cambridge, MA, USA) and GAPDH (1:3000, Abcam) overnight at 4 °C. After washing using TBST, horseradish peroxidase (HRP)-labeled secondary antibody (1:5000, Abcam) was used to incubate the membranes. Finally, all bands were examined by an ECL detection kit (Beyotime).

## In vivo experiments

To establish tumor xenografts,  $8 \times 10^6$  MGC-803 cells stably transfected with sh-circ\_0081146 or sh-NC were subcutaneously injected into the flank of BALB/c nude mice (4 weeks old). Tumor volume was measured weekly using a caliper and continuously for 5 weeks. Then, all mice were euthanized and the tumor was removed and weighed. The excised tumor tissues were applied to check the expression of circ\_0081146 by qRT-PCR. All animal experiments were approved by the Animal Care and Use Committee of Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine.

## Dual-luciferase reporter assay

Circ\_0081146 wild type (circ\_0081146-WT) or mutant type (circ\_0081146-MUT) containing miR-144 complementary binding sites or not and miR-144 mimics or miR-NC were co-transfected into AGS and MGC-803 cells using Lipofectamine 2000 (Solarbio). HMGB1 wild type (HMGB1 3'UTR-WT) with miR-144 binding sites or mutant type (HMGB1 3'UTR-MUT) without miR-144 binding sites and miR-144 mimics or miR-NC were co-transfected into AGS and MGC-803 cells. After culture for 24 h, the luciferase activity was assessed via using a Dual-luciferase reporter kit (Promega, Madison, WI, USA).

## RNA pull-down assay

The biotinylated probe was specifically designed to bind to the junction area of miR-144 and the oligo probe was used the control. Briefly, cells were reaped and lysed by lysis buffer containing protease inhibitor (Solarbio). Cell lysates were mixed with biotin-labeled miR-144 probe sequences, and incubated with streptavidin coated magnetic beads (Thermo Fisher Scientific) at 4 °C overnight. The beads were washed and the level of circ\_0081146 was quantified by qRT-PCR assay.

## RNA immunoprecipitation (RIP) assay

RIP assay was carried out using Magna RIP Kit (Millipore, Billerica, MA, USA). Briefly, AGS and MGC-803 cells were lysed by the RIP buffer supplemented with magnetic beads and then conjugated with

the anti-argonaute 2 (anti-Ago2) or IgG antibodies (negative control). Then, the protein was digested through proteinase K buffer, followed by RNA purification. Finally, qRT-PCR was performed to measure the abundance of circ\_0081146, miR-144, and HMGB1.

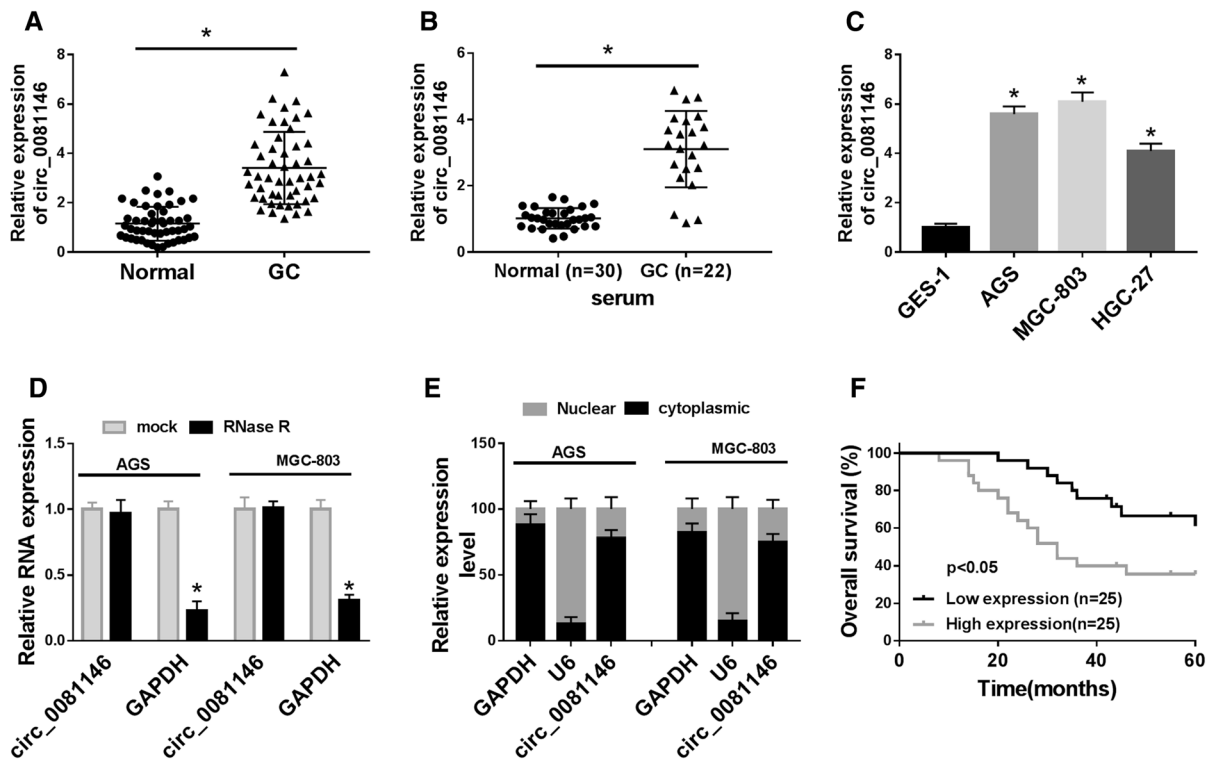
## Statistical analysis

All results were presented as mean  $\pm$  standard deviation (SD) and there were at least three independent repeats. The difference between the two sets of data was determined by Student's *t*-test. Expression correlation assay was assessed by Pearson's coefficient correlation. Differences in patient overall survival were assessed using the Kaplan–Meier method. Difference was thought to be statistically significant when *P* value was less than 0.05.

## Results

Circ\_0081146 was increased in human GC tissues and cell lines

To investigate whether circ\_0081146 was associated with GC, we first detected the expression of circ\_0081146 in 50 GC tissues and paired adjacent normal tissues. QRT-PCR results showed that circ\_0081146 expression was up-regulated in GC tissues compared to normal tissues (Fig. 1a). As expected, circ\_0081146 derived from serum was more abundant in GC patients than in healthy controls (Fig. 1b). Moreover, the expression of circ\_0081146 was significantly increased in a series of GC cell lines (AGS, MGC-803 and HGC-27) compared with a normal human gastric mucosal epithelial cell line GES-1 (Fig. 1c). To confirm the existence of circ\_0081146, the RNase R degradation assay was performed in AGS and MGC-803 cells. The results demonstrated that the linear transcripts of circ\_0081146 were degraded by RNase R treatment, while this treatment failed to degrade the circular transcripts of circ\_0081146 (Fig. 1d), the data confirmed that circ\_0081146 was a circular RNA. We then explored the localization of circ\_0081146, qRT-PCR of RNAs from nuclear and cytoplasmic fractions showed that circ\_0081146 was predominantly localized in the cytoplasm of AGS and MGC-803 cells



**Fig. 1** Circ\_0081146 was increased in human GC tissues, serum and cell lines. **a** Circ\_0081146 expression in 50 pairs of GC and noncancerous tissues was measured by qRT-PCR. **b** Circ\_0081146 expression in serum of GC patients (n = 30) and healthy controls (HC) (n = 22) was measured by qRT-PCR. **c** Circ\_0081146 expression in GC cell lines (AGS, MGC-803 and HGC-27) and gastric mucosal epithelial cell line GES-1 was

detected by qRT-PCR. **d** The level of circ\_0081146 was examined by qRT-PCR after treatment with RNase R in AGS and MGC-803 cells. **e** The level of circ\_0081146 in cytoplasm and nuclear of AGS and MGC-803 cells was detected by qRT-PCR. **f** The 5-year overall survival curve of 50 patients with GC was analyzed using Kaplan–Meier curve based on the expression of circ\_0081146. \**P* < 0.05

(Fig. 1e). Additionally, Kaplan–Meier survival curves showed that patients with higher expression of circ\_0081146 had a shorter 5-year overall survival (Fig. 1f).

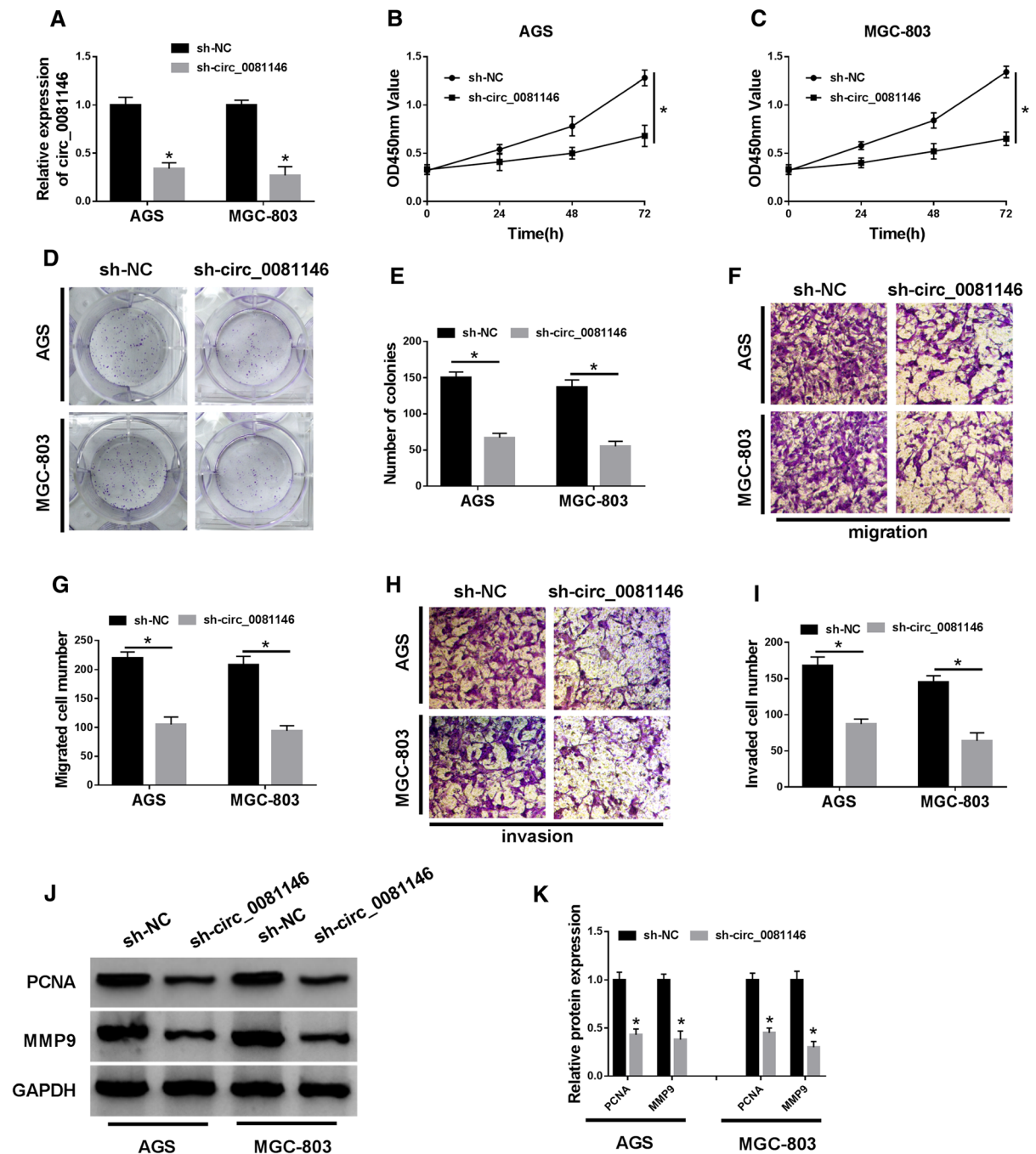
Circ\_0081146 knockdown inhibited growth, migration and invasion of GC cells in vitro

We next explored the role of circ\_0081146 in progression of GC by loss-of-function approaches. Circ\_0081146 small hairpin RNAs (shRNAs) could stably knockdown the expression of circ\_0081146 in GC cells. As shown in Fig. 2a, circ\_0081146 expression was significantly decreased by sh-circ\_0081146 in AGS and MGC-803 cells. CCK-8 assay (Fig. 2b, c) and colony formation assay (Fig. 2d, e) indicated that circ\_0081146 knockdown repressed proliferation of AGS and MGC-803 cells. Transwell assay showed

that silencing circ\_0081146 also hindered migration (Fig. 2f, g) and invasion (Fig. 2h, i) abilities of AGS and MGC-803 cells. Besides, the protein levels of PCNA and MMP9 were obviously declined in AGS and MGC-803 cells after knockdown of circ\_0081146 (Fig. 2j, k). These results indicated that circ\_0081146 knockdown impeded the progression of GC cells in vitro.

Circ\_0081146 knockdown repressed tumor growth of GC in vivo

Subsequently, we further investigated the effect of circ\_0081146 on growth of CC cells in vivo by establishing a xenograft tumor model. MGC-803 cells stably transfected with sh-circ\_0081146 or sh-NC were subcutaneously injected into the nude mice. 5 weeks after injection, we found that the tumors

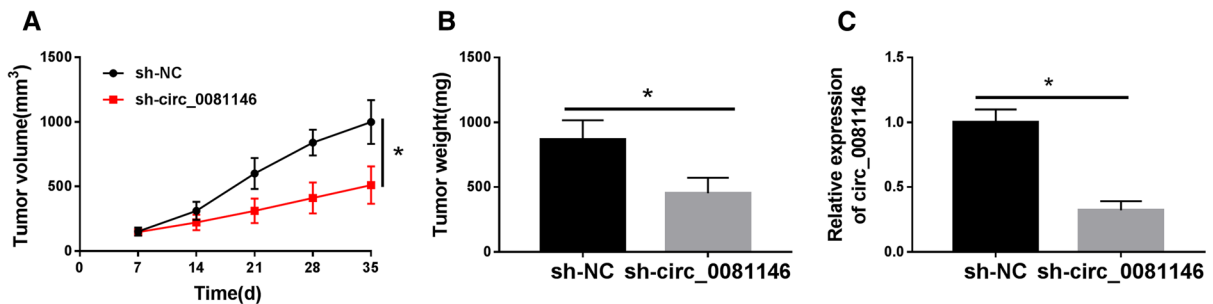


**Fig. 2** Circ\_0081146 knockdown inhibited cell growth, migration and invasion of GC cells in vitro. **a** Circ\_0081146 expression in AGS and MGC-803 cells transfected with sh-circ\_0081146 or sh-NC was examined by qRT-PCR. **b, c** The viability of AGS and MGC-803 cells was assessed by CCK-8

assay. **d, e** The proliferation of AGS and MGC-803 cells was examined by colony formation assay. **f–i** The migration and invasion of AGS and MGC-803 cells were measured by Transwell assay. **j, k** The protein levels of PCNA and MMP9 were detected by western blot. \* $P < 0.05$

generated from MGC-803 cells transfected with sh-circ\_0081146 had a smaller volume (Fig. 3a) and

lower weight (Fig. 3b). We then examined the expression of circ\_0081146 in the excised tumors by



**Fig. 3** Circ\_0081146 knockdown repressed tumor growth of GC in vivo. Stable sh-circ\_0081146 knockdown MGC-803 cells were applied for in vivo assay. **a** Tumor volume was detected

every 7 d ( $n = 8$ ). **b** The tumors were weighed after mice were euthanized. **c** The expression of circ\_0081146 was measured in that tumor tissues by qRT-PCR.  $*P < 0.05$

qRT-PCR. The data showed that circ\_0081146 expression was markedly decreased in the sh-circ\_0081146 group compared with the sh-NC group (Fig. 3c). To sum up, consistent with the in vitro data, silencing circ\_0081146 inhibited tumor growth of GC in vivo.

Circ\_0081146 acted as a sponge of miR-144 in GC cells

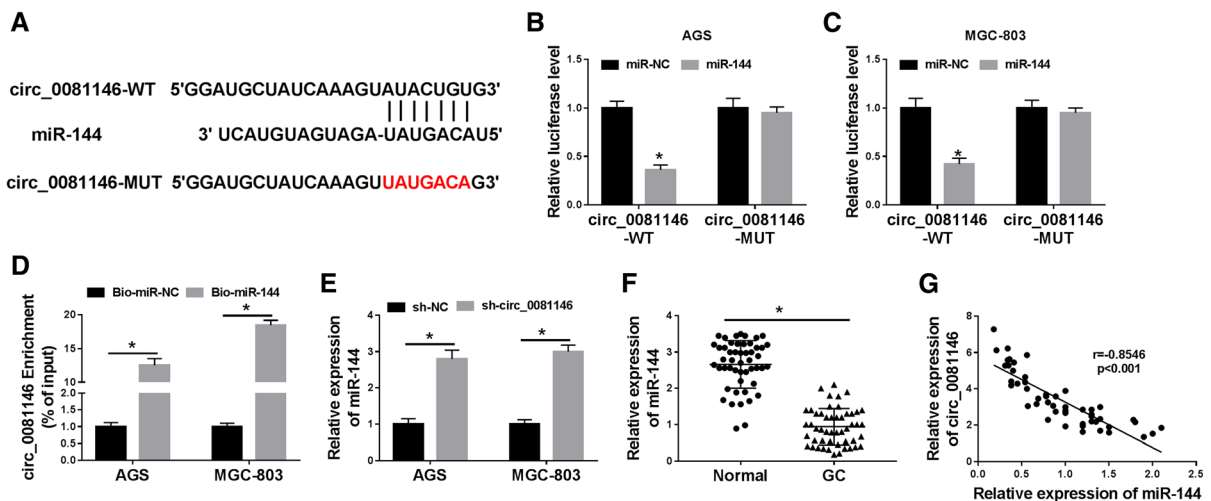
CircRNAs have been shown to function as miRNA sponges, thereby disabling the function of the corresponding miRNA (Li 2017; Yang et al. 2019). Next, we investigated whether circTADA2A affected the biological behavior of GC by sponging miRNAs. MiR-144 was predicted to be a potential target by CircInteractome, which shared complementary binding sites with circ\_0081146 (Fig. 4a). The results of dual-luciferase reporter assay indicated that miR-144 mimics (miR-144) obviously dwindled the luciferase activity of circ\_0081146-WT compared to circ\_0081146-MUT in AGS and MGC-803 cells (Fig. 4b, c), and RNA pull-down assay showed that Biotin-labeled miR-144 (Bio-miR-144) captured more circ\_0081146 compared with the biotin-labeled negative control (Bio-miR-NC) in AGS and MGC-803 cells (Fig. 4d, e). Furthermore, miR-144 expression was notably lower in GC tissues than that in normal tissues (Fig. 4f). A significant negative correlation between the levels of circ\_0081146 and miR-144 was observed in GC tissues ( $r = -0.8546$ ,  $P < 0.001$ ) (Fig. 4g). These results confirmed that circ\_0081146 could sponge miR-144 in GC cells.

Interference with miR-144 rescued the anti-tumor effect induced by sh-circ\_0081146 in GC cells

To verify whether circ\_0081146 affected the progression of GC cells by sponging miR-144, the rescue experiments were performed. We found that circ\_0081146 knockdown drastically enhanced the expression of miR-144 in AGS and MGC-803 cells, and miR-144 inhibitor (anti-miR-144) could invert the promotion effect of silencing circ\_0081146 on miR-144 expression (Fig. 5a). Functionally, the inhibitory effects of circ\_0081146 knockdown on cell proliferation (Fig. 5b, c), colony formation (Fig. 5d), migration (Fig. 5e) and invasion (Fig. 5f) were recovered by miR-144 inhibitor in AGS and MGC-803 cells. Also, the inhibition impact of circ\_0081146 deletion on protein levels of PCNA and MMP9 was regained by miR-144 inhibitor in AGS and MGC-803 cells (Fig. 5g, h). The above results demonstrated that circ\_0081146 knockdown impaired cell growth, migration and invasion of GC cells by up-regulating miR-144.

HMGB1 was validated as a target gene of miR-144

Subsequently, we predicted that miR-144 and 3' UTR of HMGB1 had binding sites by StarBase v.2.0 (Fig. 6a), and dual-luciferase reporter assay showed that miR-144 mimics enormously decreased the luciferase activity of HMGB1-WT, while there was no significant change in the luciferase activity of HMGB1-MUT in AGS and MGC-803 cells (Fig. 6b, c). In addition, RIP assay indicated that enrichment of circ\_0081146, miR-144 and HMGB1 was markedly enhanced in the Ago2 group compared with that in the



**Fig. 4** Circ\_0081146 targeted miR-144. **a** The putative binding sites of circ\_0081146 and miR-144 were predicted by CircInteractome. **b, c** The luciferase activity of AGS and MGC-803 cells co-transfected circ\_0081146-WT or circ\_0081146-MUT and miR-144 or miR-NC was checked by dual-luciferase reporter assay. **d** The biotinylated miR-144 or miR-NC was transfected into AGS and MGC-803 cells and the level of

circ\_0081146 was quantified by qRT-PCR. **e** The expression of miR-144 in AGS and MGC-803 cells transfected with sh-circ\_0081146 or sh-NC was examined by qRT-PCR. **f** MiR-144 expression in GC and noncancerous tissues was checked by qRT-PCR. **g** The correlation between circ\_0081146 and miR-144 expression ( $r = -0.8546$ ,  $P < 0.001$ ) was assessed by Pearson's coefficient correlation.  $*P < 0.05$

IgG group (Fig. 6d, e). More than that, miR-144 overexpression down-regulated the mRNA and protein levels of HMGB1 in AGS and MGC-803 cells (Fig. 6f–h). To explore whether HMGB1 expression was related to GC, its expression in GC tissues was examined. The data of western blot assays showed that the protein level of HMGB1 was increased in GC tissues (Fig. 6i). More importantly, HMGB1 expression was negatively correlated with the miR-144 expression in GC tissues ( $r = -0.6905$ ,  $P < 0.001$ ) (Fig. 6j), while the expression of HMGB1 was positively associated with the expression of circ\_0081146 ( $r = 0.5963$ ,  $P < 0.001$ ) (Fig. 6k).

Overexpression of HMGB1 reversed the effect of silencing circ\_0081146 on GC cells

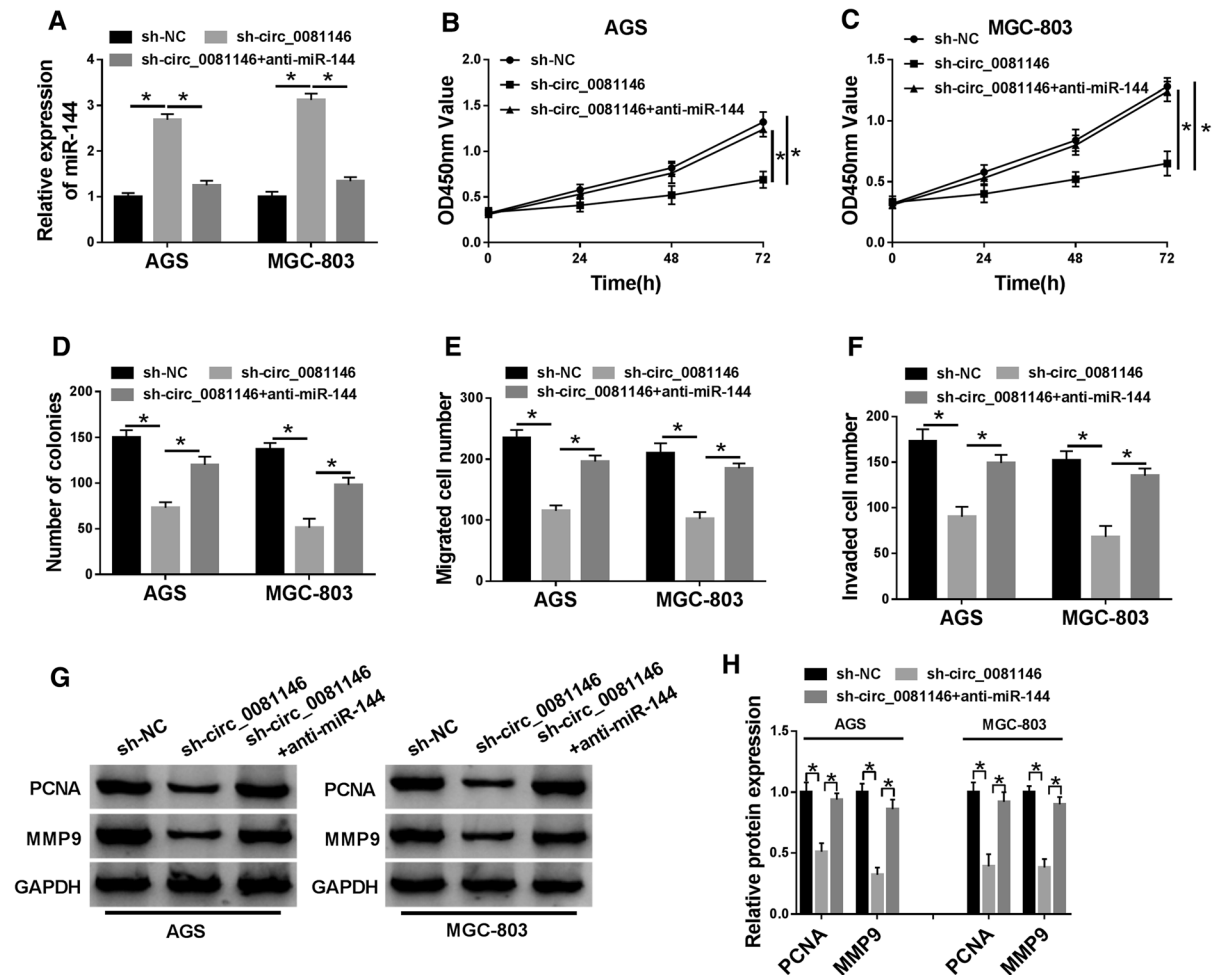
Considering that circ\_0081146 directly targeted miR-144 and HMGB1 was the target gene of miR-144, we further investigated whether circ\_0081146 could affect the progress of GC cells by regulating HMGB1. Firstly, the mRNA and protein levels of HMGB1 were silenced in AGS and MGC-803 cells transfected with sh-circ\_0081146, and overexpression of HMGB1 could overturn the effect of sh-circ\_0081146 on HMGB1 expression (Fig. 7a–c). Meanwhile, we

found that overexpression of HMGB1 in AGS and MGC-803 cells reversed the inhibitory impact of circ\_0081146 knockdown on cell proliferation (Fig. 7d, e), colony formation (Fig. 7f), migration (Fig. 7g), invasion (Fig. 7h), and protein levels of PCNA and MMP9 (Fig. 7i). These results indicated that knockdown of circ\_0081146 inhibited growth, migration and invasion of GC cells by regulating the expression of HMGB1.

Circ\_0081146 up-regulated the expression of HMGB1 by targeting miR-144 in GC cells

To confirm whether circ\_0081146 could regulate HMGB1 expression by targeting miR-144, AGS and MGC-803 cells were transfected with sh-NC, sh-circ\_0081146, sh-circ\_0081146 + anti-miR-144. The results demonstrated that circ\_0081146 knockdown distinctly reduced the mRNA and protein levels of HMGB1, and interference with miR-144 could reverse the impact of circ\_0081146 knockdown on HMGB1 expression (Fig. 8a–c), signifying that circ\_0081146 acted as a ceRNA of miR-144 to increase the expression of HMGB1 in GC cells.





**Fig. 5** Interference with miR-144 rescued the anti-tumor effect induced by sh-circ\_0081146 in GC cells. **a** MiR-144 expression in AGS and MGC-803 cells transfected with sh-NC, sh-circ\_0081146, sh-circ\_0081146 + anti-miR-144 was detected by qRT-PCR. **b, c** The viability of AGS and MGC-803 cells was

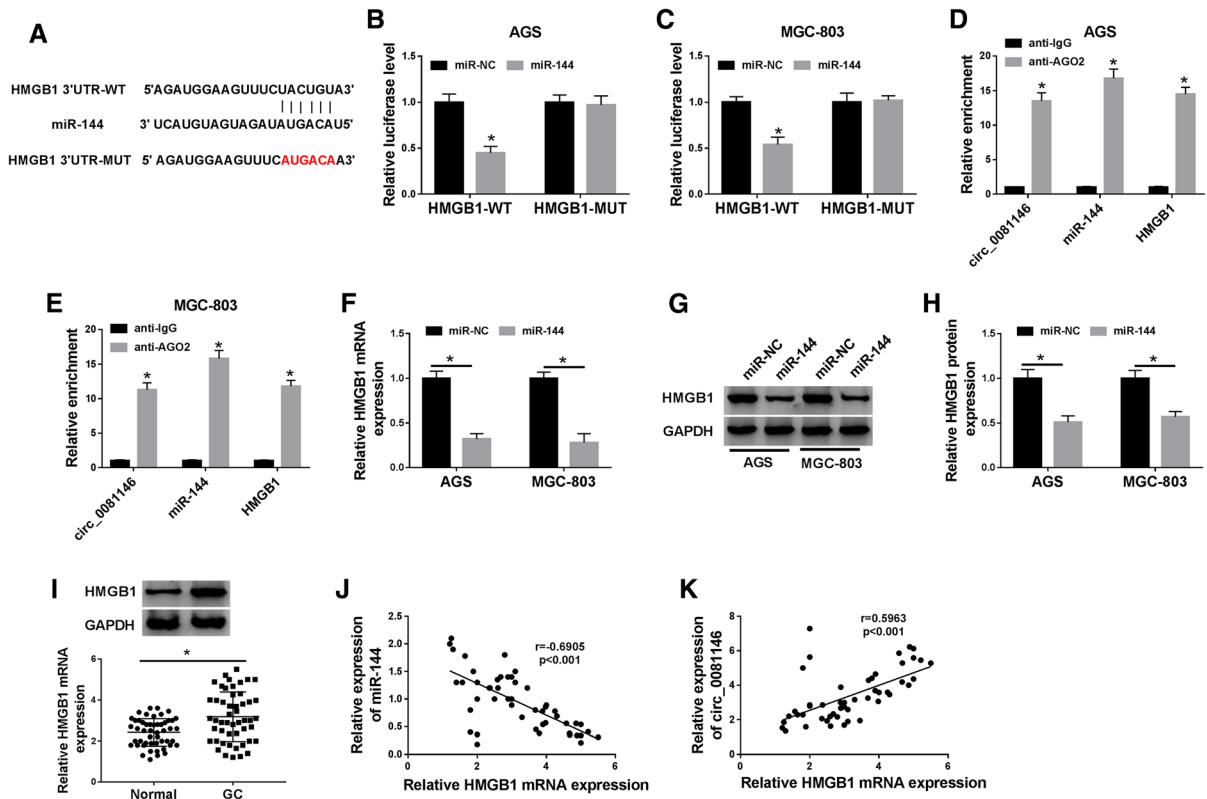
examined by CCK-8 assay. **d** The proliferation of AGS and MGC-803 cells was measured by colony formation assay. **e, f** The migration and invasion of AGS and MGC-803 cells were assessed by Transwell assay. **g, h** The protein levels of PCNA and MMP9 were measured by western blot. \* $P < 0.05$

**Discussion**

As a type of ncRNAs, the expression pattern of circRNAs has attracted attention (Vicens and Westhof 2014). CircRNAs are specifically expressed in cancer tissues and involved in regulating the development of cancer, which may offer a novel approach for cancer treatment (Chen et al. 2016). In this study, we found that circ\_0081146 was augmented in GC tissues, serum and cells, which was consistent with the previous microarray data (Dang et al. 2017). In addition, circ\_0081146 showed little degradation after treatment with RNase R, demonstrating the stability of

circRNA reported in previous studies (Wu et al. 2018). Simultaneously, circ\_0081146 was confirmed to be an exon circRNA mainly expressed in the cytoplasm of GC cells, and the high expression of circ\_0081146 could reduce the 5-year overall survival rate of GC patients. In terms of function, we found that silencing circ\_0081146 hindered growth, migration and invasion of GC cells in vitro, and impeded the growth of tumor in vivo. These results suggested that circ\_0081146 might be a prognostic marker as an oncogene in GC.

There are a variety of miRNAs binding sites in circRNAs, and it has been well documented that



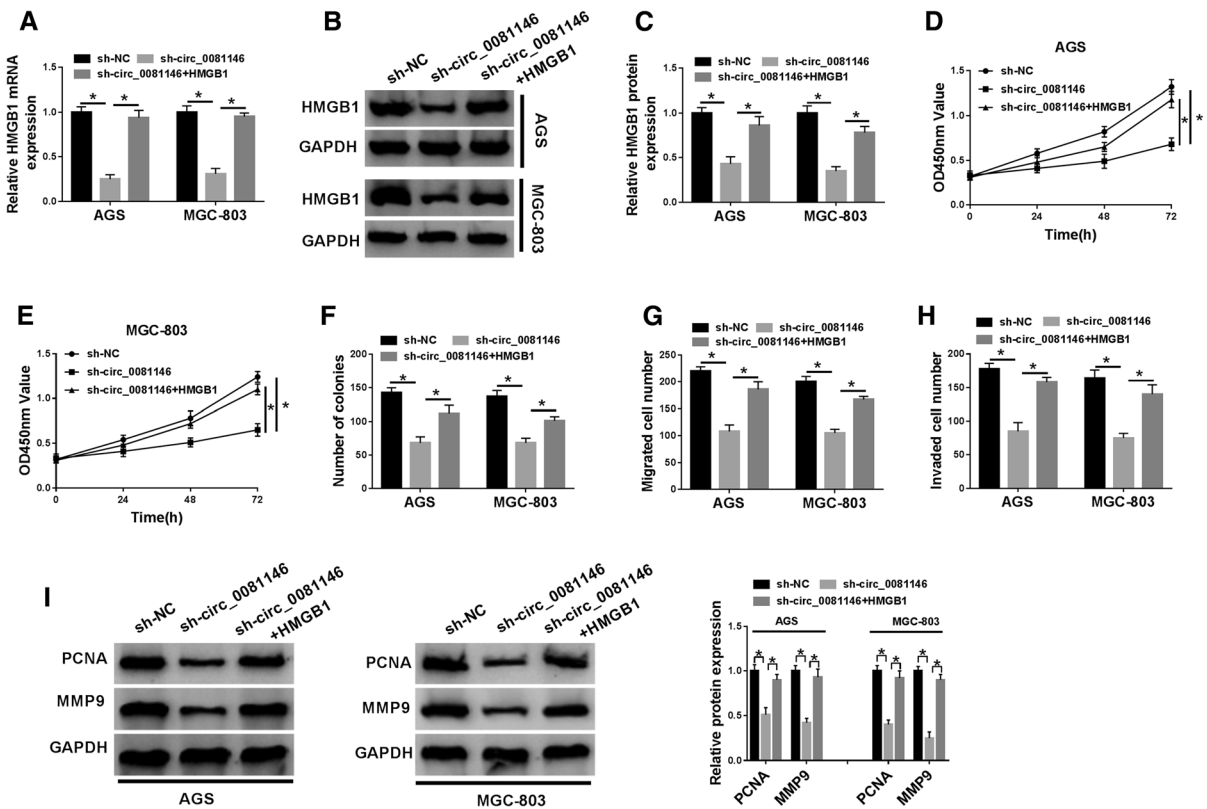
**Fig. 6** HMGB1 was a target gene of miR-144. **a** The putative binding sites of miR-144 and HMGB1 3'UTR were predicted by StarBase v.2.0. **b, c** The luciferase activity of AGS and MGC-803 cells co-transfected HMGB1-WT or HMGB1-MUT and miR-144 or miR-NC was measured by dual-luciferase reporter assay. **d, e** The enrichment of circ\_0081146, miR-144 or HMGB1 was measured by RIP assay in AGS and MGC-803 cells incubated with Ago2 or IgG. **f–h** The mRNA and protein

levels of HMGB1 in AGS and MGC-803 cells transfected with miR-144 or miR-NC were examined by qRT-PCR and western blot, respectively. **i** The protein level of HMGB1 in GC tissues and normal tissues was detected by western blot. **j, k** The correlations between HMGB1 and miR-144 expression ( $r = -0.6905$ ,  $P < 0.001$ ), and HMGB1 and circ\_0081146 expression ( $r = 0.5963$ ,  $P < 0.001$ ) were analyzed by Pearson's coefficient correlation. \* $P < 0.05$

circRNAs modulate the expression of target mRNAs by serving as competitive endogenous RNAs (ceRNAs) to sponge miRNAs (Gong et al. 2019). Furthermore, the miRNA-mRNA axis has been found to be regulated by circRNA in a variety of tumor cells, thereby promoting or inhibiting cancer progression (Chan and Tay 2018). For example, circRNA CACTIN facilitated the progression of GC via miR-331-3p/TGFBR1 regulatory axis (Zhang et al. 2019). In this study, we validated that circ\_0081146 could target miR-144 in GC cells through dual-luciferase reporter and RNA pull down assays. MiR-144 has been found to be down-regulated in a variety of cancers, including colorectal cancer (Shi et al. 2019) and osteosarcoma (Liu et al. 2019). MiR-144 has also been shown to inhibit the progression of GC by

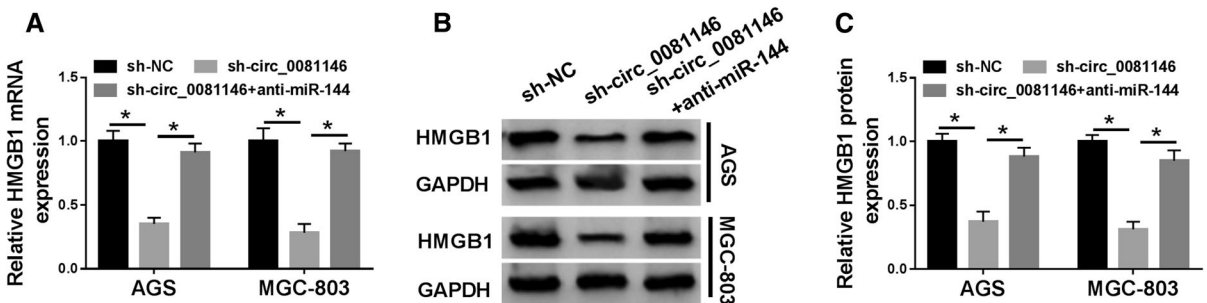
targeting PIM1 (Ren et al. 2017) and COX-2 (Yao et al. 2018), respectively. However, a study reported that miR-144 acted as an oncogene in nasopharyngeal carcinoma to promote cancer development (Zhang et al. 2013). Our data showed that miR-144 was declined in GC tissues. Moreover, circ\_0081146 knockdown suppressed growth, migration and invasion of GC cells through up-modulating miR-144 expression, this also proved that miR-144 was an anti-tumor factor in GC, which was consistent with previous data (Liu et al. 2015).

According to previous research reports, abnormally expressed HMGB1 is related to the occurrence and development of many cancers (Tripathi et al. 2019). Specifically, HMGB1 could accelerate angiogenesis and tumor migration in breast cancer (He et al. 2019).



**Fig. 7** Overexpression of HMGB1 reversed the effect of silencing of circ\_0081146 on GC cells. **a–c** The mRNA and protein levels of HMGB1 in AGS and MGC-803 cells transfected with sh-NC, sh-circ\_0081146, sh-circ\_0081146 + HMGB1 were detected by qRT-PCR and western blot, respectively. **d–f** The viability and proliferation of AGS and

MGC-803 cells were assessed by CCK-8 and colony formation assays, respectively. **g, h** The migration and invasion of AGS and MGC-803 cells were measured by Transwell assay. **i** PCNA and MMP9 protein levels were checked by western blot. \**P* < 0.05



**Fig. 8** Circ\_0081146 up-regulated the expression of HMGB1 by targeting miR-144 in GC cells. **a–c** The mRNA and protein levels of HMGB1 in AGS and MGC-803 cells transfected with

sh-NC, sh-circ\_0081146, sh-circ\_0081146 + anti-miR-144 were detected by qRT-PCR and western blot, respectively. \**P* < 0.05

Zhang et al. indicated that HMGB1 release mediated by autophagy promoted cell survival in GC (Zhang et al. 2015). Our results suggested that HMGB1 was directly targeted by miR-144 and a significant inverse

correlation between them was also observed. More than that, the expression of HMGB1 was actively related to the circ\_0081146 expression. In the functional assays, overexpression of HMGB1 eliminated

the inhibition effect of circ\_0081146 knockdown on GC cells. Interestingly, circ\_0081146 could serve as a ceRNA of miR-144 to increase the expression of HMGB1 in GC cells. To sum up, these data implied that circ\_0081146 knockdown repressed the progression of GC via acting as a miR-144 sponge to modulate HMGB1 expression.

In conclusion, our data supported that circ\_0081146 was up-regulated in GC tissues, serum and cells, and the high expression of circ\_0081146 was related to the low 5-year overall survival rate of GC patients. Additionally, the growth, migration and invasion abilities of GC cells were restrained through interference with circ\_0081146. Our results revealed that circ\_0081146 might up-regulate the expression HMGB1 to facilitate the progress of GC by sponging miR-144, providing a novel molecular mechanism for the study of GC development.

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#### Compliance with ethical standards

**Conflict of interest** The authors have no conflict of interest to declare.

#### References

- Ambros V (2001) microRNAs: tiny regulators with great potential. *Cell* 107:823–826. [https://doi.org/10.1016/S0092-8674\(01\)00616-X](https://doi.org/10.1016/S0092-8674(01)00616-X)
- Chan JJ, Tay Y (2018) Noncoding RNA:RNA regulatory networks in cancer. *Int J Mol Sci* 19:1310. <https://doi.org/10.3390/ijms19051310>
- Chen Y, Li C, Tan C, Liu X (2016) Circular RNAs: a new frontier in the study of human diseases. *J Med Genet* 53:359–365. <https://doi.org/10.1136/jmedgenet-2016-103758>
- Chen J et al (2017) Circular RNA profile identifies circPVT1 as a proliferative factor and prognostic marker in gastric cancer. *Cancer Lett* 388:208–219. <https://doi.org/10.1016/j.canlet.2016.12.006>
- Dang Y et al (2017) Circular RNAs expression profiles in human gastric cancer. *Sci Rep* 7:9060. <https://doi.org/10.1038/s41598-017-09076-6>
- D'Angelo B et al (2016) MicroRNAs: a puzzling tool in cancer diagnostics and therapy. *Anticancer Res* 36:11:5571–5575. <https://doi.org/10.21873/anticancer.11142>
- Ge S et al (2019) MiR-181a, a new regulator of TGF- $\beta$  signaling, can promote cell migration and proliferation in gastric cancer. *Invest New Drugs* 37:923–934. <https://doi.org/10.1007/s10637-018-0695-5>
- Gong J et al (2019) Integrated analysis of circular RNA-associated ceRNA network in cervical cancer: observational study. *Medicine (Baltimore)* 98:e16922. <https://doi.org/10.1097/md.00000000000016922>
- He H, Wang X, Chen J, Sun L, Sun H, Xie K (2019) High-Mobility Group Box 1 (HMGB1) promotes angiogenesis and tumor migration by regulating hypoxia-inducible factor 1 (HIF-1 $\alpha$ ) expression via the phosphatidylinositol 3-Kinase (PI3K)/AKT signaling pathway in breast cancer cells. *Med Sci Monit* 25:2352–2360. <https://doi.org/10.12659/msm.915690>
- Huang S et al (2019a) A novel circular RNA hsa\_circ\_0008035 contributes to gastric cancer tumorigenesis through targeting the miR-375/YBX1 axis. *Am J Transl Res* 11:2455–2462
- Huang X et al (2019b) Circular RNA AKT3 upregulates PIK3R1 to enhance cisplatin resistance in gastric cancer via miR-198 suppression. *Mol Cancer* 18:71. <https://doi.org/10.1186/s12943-019-0969-3>
- Jin W et al (2019) Downregulation miR-539 is associated with poor prognosis of gastric cancer patients and aggressive progression of gastric cancer cells. *Cancer Biomark* 26:183–191. <https://doi.org/10.3233/CBM-190384>
- Karimi P, Islami F, Anandasabapathy S, Freedman ND, Kamangar F (2014) Gastric cancer: descriptive epidemiology, risk factors, screening, and prevention. *Cancer Epidemiol Biomarkers Prev* 23:700–713. <https://doi.org/10.1158/1055-9965.epi-13-1057>
- Ke XS, Liu CM, Liu DP, Liang CC (2003) MicroRNAs: key participants in gene regulatory networks. *Curr Opin Chem Biol* 7:516–523. [https://doi.org/10.1016/S1367-5931\(03\)00075-9](https://doi.org/10.1016/S1367-5931(03)00075-9)
- Ledford H (2013) Circular RNAs throw genetics for a loop. *Nature* 494:415. <https://doi.org/10.1038/494415a>
- Li Y et al (2015) Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. *Cell Res* 25:981–984. <https://doi.org/10.1038/cr.2015.82>
- Li Y et al (2017) CircHIPK3 sponges miR-558 to suppress heparanase expression in bladder cancer cells. *EMBO Rep* 18:1646–1659. <https://doi.org/10.15252/embr.201643581>
- Li P, Xu M, Cai H, Thapa N, He C, Song Z (2019) The effect of HMGB1 on the clinicopathological and prognostic features of cervical cancer. *Biosci Rep*. <https://doi.org/10.1042/bsr20181016>
- Liu J et al (2015) MicroRNA-144 inhibits the metastasis of gastric cancer by targeting MET expression. *J Exp Clin Cancer Res* 34:35. <https://doi.org/10.1186/s13046-015-0154-5>
- Liu S et al (2017) Prognostic significance of low miR-144 expression in gastric cancer. *Cancer Biomark* 204:547–552. <https://doi.org/10.3233/CBM-170351>
- Liu H et al (2018) Circular RNA YAPI inhibits the proliferation and invasion of gastric cancer cells by regulating the miR-367-5p/p27 (Kip1) axis. *Mol Cancer* 17:151. <https://doi.org/10.1186/s12943-018-0902-1>
- Liu JL et al (2019) MiR-144 inhibits tumor growth and metastasis in osteosarcoma via dual-suppressing RhoA/ROCK1 signaling pathway. *Mol Pharmacol* 95:451–461. <https://doi.org/10.1124/mol.118.114207>

- Lv DJ et al (2019) HMGB1 promotes prostate cancer development and metastasis by interacting with brahma-related gene 1 and activating the Akt signaling pathway. *Theranostics* 9:5166–5182. <https://doi.org/10.7150/thno.33972>
- Miao Z et al (2019) The long noncoding RNA NORAD promotes the growth of gastric cancer cells by sponging miR-608. *Gene* 687:116–124. <https://doi.org/10.1016/j.gene.2018.11.052>
- Ren K, Liu QQ, An ZF, Zhang DP, Chen XH (2017) MiR-144 functions as tumor suppressor by targeting PIM1 in gastric cancer. *Eur Rev Med Pharmacol Sci* 21:3028–3037
- Shi L et al (2019) Long non-coding RNA ZNFX1-AS1 promotes the tumor progression and metastasis of colorectal cancer by acting as a competing endogenous RNA of miR-144 to regulate EZH2 expression. *Cell Death Dis* 10:150. <https://doi.org/10.1038/s41419-019-1332-8>
- Suren D, Arda Gokay A, Sayiner A (2018) High Mobility Group Box 1 (HMGB1) expression in gastric adenocarcinomas. *J Buon* 23:422–427
- Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A (2015) Global cancer statistics, 2012. *CA Cancer J Clin* 65:87–108. <https://doi.org/10.3322/caac.21262>
- Tripathi A, Shrinet K, Kumar A (2019) HMGB1 protein as a novel target for cancer. *Toxicol Rep* 6:253–261. <https://doi.org/10.1016/j.toxrep.2019.03.002>
- Vicens Q, Westhof E (2014) Biogenesis of circular RNAs. *Cell* 159:13–14. <https://doi.org/10.1016/j.cell.2014.09.005>
- Wu J et al (2018) CircIRAK3 sponges miR-3607 to facilitate breast cancer metastasis. *Cancer Lett* 430:179–192. <https://doi.org/10.1016/j.canlet.2018.05.033>
- Yang H, Wang H, Czura CJ, Tracey KJ (2005) The cytokine activity of HMGB1. *J Leukoc Biol* 78:1–8. <https://doi.org/10.1189/jlb.1104648>
- Yang J et al (2019) Circular RNA hsa\_circRNA\_0007334 is predicted to promote MMP7 and COL1A1 expression by functioning as a miRNA sponge in pancreatic ductal adenocarcinoma. *J Oncol* 2019:7630894. <https://doi.org/10.1155/2019/7630894>
- Yao Q, Gu A, Wang Z, Xue Y (2018) MicroRNA-144 functions as a tumor suppressor in gastric cancer by targeting cyclooxygenase-2. *Exp Ther Med* 15:3088–3095. <https://doi.org/10.3892/etm.2018.5763>
- Zhang LY et al (2013) MicroRNA-144 promotes cell proliferation, migration and invasion in nasopharyngeal carcinoma through repression of PTEN. *Carcinogenesis* 34:454–463. <https://doi.org/10.1093/carcin/bgs346>
- Zhang J, Kou YB, Zhu JS, Chen WX, Li S (2014) Knockdown of HMGB1 inhibits growth and invasion of gastric cancer cells through the NF-kappaB pathway in vitro and in vivo. *Int J Oncol* 44:1268–1276. <https://doi.org/10.3892/ijo.2014.2285>
- Zhang QY, Wu LQ, Zhang T, Han YF, Lin X (2015) Autophagy-mediated HMGB1 release promotes gastric cancer cell survival via RAGE activation of extracellular signal-regulated kinases 1/2. *Oncol Rep* 33:1630–1638. <https://doi.org/10.3892/or.2015.3782>
- Zhang J et al (2017) Circular RNA\_LARP4 inhibits cell proliferation and invasion of gastric cancer by sponging miR-424-5p and regulating LATS1 expression. *Mol Cancer* 16:151. <https://doi.org/10.1186/s12943-017-0719-3>
- Zhang J et al (2018) High mobility group box 1 promotes the epithelial-to-mesenchymal transition in prostate cancer PC3 cells via the RAGE/NF-kappaB signaling pathway. *Int J Oncol* 53:659–671. <https://doi.org/10.3892/ijo.2018.4420>
- Zhang L, Song X, Chen X, Wang Q, Zheng X, Wu C, Jiang J (2019) Circular RNA CircCACTIN promotes gastric cancer progression by sponging MiR-331-3p and regulating TGFBR1 expression. *Int J Biol Sci* 15:1091–1103. <https://doi.org/10.7150/ijbs.31533>

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