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LncRNA NR2F2-AS1 functions as a tumor suppressor in gastric cancer through targeting miR-320b/PDCD4 pathway

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Summary. Gastric cancer is among the most frequently occurring gastrointestinal malignancies with a high mortality rate worldwide. Long non-coding RNAs (lncRNAs) are defined as core regulators in the occurrence and progression of multiple cancers, including gastric carcinoma. Mounting evidence has indicated that NR2F2-AS1 can inhibit several malignant tumors. However, the function and potential mechanism of NR2F2-AS1 remain unclear. In the current study, we found that NR2F2-AS1 was weakly expressed in gastric cancer cells in comparison with normal cells. The study has further disclosed that ectopic of NR2F2-AS1 repressed cell proliferation, migration, invasion and EMT whereas it promoted cell apoptosis in gastric carcinoma. Subsequently, our results confirmed that miR-320b was negatively regulated and that suppression of miR-320b alleviated the malignant behaviors of GC cells. More importantly, PDCD4 was a target of miR-320b. Mechanistically, NR2F2-AS1 modulated the expression level of PDCD4 by sponging miR-320b. Finally, rescue assays demonstrated that NR2F2-AS1 down-regulated PDCD4 expression to restrain the development of gastric cancer by competitively binding to miR-320b. On the whole, our study revealed the role of NR2F2-AS1/miR-320b/PDCD4 regulatory network in gastric cancer, suggesting NR2F2-AS1 may represent a novel therapeutic target for patients with gastric carcinoma.

Key words: Gastric cancer, NR2F2-AS1, miR-320b, PDCD4

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Introduction

As a frequently diagnosed malignancy, gastric cancer (GC) is the fourth leading cause of cancer-related mortality and accounts for roughly 5.6% of new cases and 7.7% of deaths worldwide (Sung et al., 2021). A great number of risk factors contribute to GC, including smoking and helicobacter pylori infection. Seniors and males are also vulnerable to GC (Machlowska et al., 2020). It is estimated that two-thirds of GC patients are in advanced stage at their initial diagnosis with a 5-year overall survival rate of about 20%-30% (Li et al., 2020b). The lack of accurate diagnosis in GC is the main contributor to death. Regarding this, it is imperative to find specific markers in the diagnosis of GC and comprehend its potential mechanism.

Long non-coding RNAs (lncRNAs) are regarded as a category of non-protein coding RNA consisting of more than 200 nucleotides (Wei et al., 2020). Recently, lncRNAs have drawn increasing attention, and a growing body of research has revealed the role of lncRNAs in various diseases. Multiple lines of evidence demonstrate that lncRNAs are involved in regulating the epigenetic, transcriptional or post-transcriptional expression of numerous target genes by functioning as scaffolds or miRNA sponges (Li et al., 2020a). It is widely acknowledged that aberrantly expressed lncRNAs play important roles in the initiation and development of malignancies, including GC. Dysregulated lncRNAs has shown to work as oncogenes or tumor suppressors through affecting a wide spectrum of biological activities, such as cell growth, metastasis, drug resistance and autophagy (Lin et al., 2018; Bermudez et al., 2019). For example, high expression of the long non-coding RNA FOXD2-AS1 contributes to tumor progression and metastasis of papillary thyroid cancer (Li et al., 2019). Long non-coding RNA TPT1-AS1 alleviates the microRNA-770-5p expression to suppress glioma cell autophagy and facilitate proliferation through upregulation of STMN1 (Jia et al., 2020). LncRNA SNHG7 promotes cell growth and represses apoptosis in gastric cancer by inhibition of P15

and P16 expression (Wang et al., 2017).

LncRNA NR2F2-AS1, also known as COUP-TFII, belongs to the nuclear receptor family, which is located at the 15q26.2 site on the chromosome and transcribes the transcript of 1161nt. It has been reported to participate in the occurrence and development of multiple tumors. The expression of NR2F2-AS1 is up-regulated in cervical cancer tissues and cells, especially in patients with advanced cervical cancer. Furthermore, NR2F2-AS1 accelerates cervical cancer progression, promoting cell proliferation, migration, invasion, and EMT by sponging miR-4429 to increase the expression of oncogene MBD1 (Liu et al., 2020a); NR2F2-AS1 was up-regulated in CRC, and CRC patients with high levels of NR2F2-AS1 showed a low overall survival rate. Besides, the expression of Cyclin D1 was positively correlated with NR2F2-AS1, NR2F2-AS1 silencing mediates the downregulation of Cyclin D1 to induce G0/G1 arrest in colorectal cancer (Liu et al., 2020b). In spite of this, the potential of NR2F2-AS1 in the tumorigenesis of GC is still unknown.

Herein, we propose to shed light on the expression pattern and functional role of NR2F2-AS1 in GC. Our data showed that low expression of NR2F2-AS1 was observed in clinical samples and cells of GC, which predicted the poor outcomes of GC. Gain of function assays unraveled that NR2F2-AS1 restrained the progression of GC through affecting cell proliferation, migration, invasion and EMT. To further follow up on the regulatory mechanism of NR2F2-AS1, we prospected the downstream target of NR2F2-AS1. Mechanistically, NR2F2-AS1 executed tumor suppressor properties in GC by elevating the expression of PDCD4 by sponging miR-320b.

Materials and methods

Tissue samples

50 paired tumor samples and adjacent normal tissues were collected from patients who were diagnosed with GC in The Second Xiangya Hospital. All clinical tissues were obtained under the approval of the Ethics Committee of The Second Xiangya Hospital (IRB2019-S090). Written informed consents were obtained from all GC patients enrolled in the study. No participants received any radiotherapy or chemotherapy prior to resection surgery. This study was conducted in accordance with the Declaration of Helsinki. Samples were frozen with liquid nitrogen and stored at -80□ for subsequent use.

Cell culture

GC cells (AGS, MGC-803, SGC-7901 and SNU-1) and human normal gastric epitheliums GES-1 as well as HEK 293T cells were procured from the Chinese Academy of Medical Science (Shanghai, China). GC cells and GES-1 cells were cultivated in a culture medium consisting of RPMI-1640 medium (Gibco, Rockville, MD, USA), 10% FBS (Gibco), 100 μg/mL streptomycin and 100 U/mL penicillin. HEK 293T cells were grown in a DMEM medium (Hyclone, South Logan, UT, USA) containing 10% FBS (Gibco). All cells were cultured at 37°C in the presence of 5% CO_2.

Cell transfection

The full length of NR2F2-AS1 was ligated in the pcDNA3.1 vector bought from GenePharma (Shanghai, China) with an empty plasmid as a negative control. To regulate miR-320b expression, miR-320b mimic and inhibitor, as well as their matched scrambled RNAs as negative controls (NC mimic and NC inhibitor) were also supplied by Gene Pharma. For knockdown of PDCD4, shRNAs against PDCD4 (sh-PDCD4) and negative control shRNAs (sh-NC) were acquired from RiboBio (Guangzhou, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was adopted for cell transfection in conformity with the directions of the manufacturer. The cells were collected at 48 h post transfection.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from clinical samples and cells with Trizol reagent (Invitrogen). The cDNA was generated by PrimeScript RT reagent Kit (Takara, Ohtsu, Japan). Real-time PCR was performed with the KAPA PCR kit (Sigma-Aldrich, St. Louis, MO, USA) in line with the supplier's recommendations. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expression. The primer sequences for PCR were displayed as follow: for NR2F2-AS1: 5'-TCAGCCGGAAAACTACAAGCTC-3' (forward) and 5'-TCTTCGTGTAGCTGTTCCACC-3' (reverse); for miR-320b: 5'- GCAAAAGCTGGGT TGAGA-3' (forward) and 5'-CAGTGCGTGTCGTG GA-3' (reverse); for PDCD4: 5'-GGCCTCCAAGGA GTAAGACC-3' (forward) and 5'-AGGGGTCTACAT GGCAACTG-3' (reverse); for GAPDH: 5'-TATGATGATATCAAGAGGGTAGT-3' (forward) and 5'-TGTATCCAAACTCATTGTCATAC-3' (reverse); for U6: 5'-CTCGCTTCGGCAGCACA-3' (forward) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse). GAPDH and U6 were employed as internal references for the normalization of genes.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan) was used for the detection of cell proliferation. After transfection, 2×10^3 AGS and MGC-803 cells were inoculated into each well of a 96-well plate. At 0, 24, 48 and 72 h post incubation, cells were processed with CCK-8 reagent, followed by incubation for additional

2 h at 37°C. Then they underwent absorbance measurement at 450 nm.

For colony formation assay, 1×10^3 transfected AGS and MGC-803 cells were seeded to 6-well plates and cultured in RPMI-1640 medium. 2 weeks later, cells were washed twice in PBS and fixed in methanol. they were then stained with 0.1% crystal violet solution (Sigma, St. Louis, MO, USA). Subsequently, visible colonies were counted.

TUNEL staining

Cell apoptosis was monitored by TUNEL assay using the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN, USA) according to the guidelines of the manufacturer. After transfection, AGS and MGC-803 cells were immobilized in 4% paraformaldehyde, followed by permeabilization with 0.1% Triton X-100, stained by TUNEL reaction solution, and then redyed with DAPI. TUNEL-positive cells were counted under a fluorescence microscope (Thermo Fisher Scientific, Boston, MA, USA).

Transwell assays

The migratory and invasive abilities of AGS and MGC-803 cells were estimated by transwell assays. In short, transfected cells were seeded on the upper chamber of 8 μm pore size membranes containing serum-free medium. RPMI-1640 medium with 10% FBS was added to the bottom chamber. 24 h later, cells on the surface of membranes were wiped. Migrated cells were fixed in 10% formaldehyde, dyed with 0.1% crystal violet and then counted with a light microscope (Olympus, Tokyo, Japan). To evaluate cell invasion, all the steps were performed similarly to those in transwell migration assay except for the Matrigel (BD Bioscience, Detroit, MI, USA) coating.

Western blot

RIPA buffer (Thermo Fisher Scientifc, Inc., Wilmington, DE, USA) was adopted to extract total protein from AGS and MGC-803 cells. After protein quantification, the equivalent amount of total protein was segregated on SDS-PAGE and subsequently transferred to PVDF membranes (Millipore, Billerica, MA, USA). Thereafter, the membranes were blocked in 5% skimmed milk, undergoing incubation with primary antibody at 4°C overnight. Then they were probed by secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h, followed by measurement with ECL detection kit (Thermo Fisher Scientifc, Inc.) on ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA). The primary antibodies against PDCD4, E-cadherin, Vimentin and GAPDH were all obtained from Cell Signaling Technology. The expression of proteins was normalized to GAPDH.

RNA pull down assay

AGS and MGC-803 cells were transfected with biotin-labeled miRNA: wild-type miR-320b (Bio-miR-320b-WT), mutant miR-320b without PDCD4 binding sites (Bio-miR-320b-Mut), negative control Bio-NC. After transfection, the lysates from AGS and MGC-803 cells underwent incubation with M-280 streptavidin magnetic beads (Invitrogen). Bound RNAs were eluted from beads with RNase-free DNase I (Roche Diagnostics) and then purified with RNeasy Mini Kit (Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's directions. The abundance of PDCD4 in bound RNAs was examined by qRT-PCR analysis.

RNA immunoprecipitation (RIP)

To confirm the interplay between NR2F2-AS1 and miR-320b, RIP Assay Kit (Millipore, Billerica, MA, USA) was used to carry out the RIP experiment following the recommendations of the suppliers. In brief, harvested AGS and MGC-803 cells were resuspended in RIP lysis buffer and therewith went through overnight incubation with RIP buffer containing magnetic beads coated with anti-Ago2 antibody or negative control anti-IgG antibody at 4°C. Having been washed three times, beads were treated with proteinase K and then bound RNAs were isolated from beads. The qRT-PCR assay was implemented to detect the relative expression of NR2F2-AS1 and miR-320b.

Luciferase reporter assay

The StarBase database (version 2.0; http://starbase.sysu.edu.cn/starbase2) was used to predict the potential miRNA and miR-320b target genes. The 3'UTR of NR2F2-AS1 containing binding sites for miR-320b were cloned into pGL3 reporter vector (Promega, Madison, WI, USA) to shape NR2F2-AS1-WT or NR2F2-AS1-Mut respectively. PDCD4-WT and PDCD4-Mut reporter plasmids were constructed in the same manner. HEK 293T cells were co-transfected with corresponding vectors and miR-320b mimic or NC mimic. Dual-Luciferase Reporter Assay System (Promega) was employed to detect luciferase activity in 24 h post transfection.

Statistical analysis

All data are shown as means ± SD, and all experiments were independently conducted at least three times. Statistical analyses were carried out by using SPSS version 21.0 software. Student's t test and one-way ANOVA were applied for comparison between two groups or more. Overall survival rate was analyzed by Kaplan-Meier method along with the log-rank test. The association between NR2F2-AS1, miR-320b and PDCD4 was assessed by Pearson's correlation analysis.

Statistically significant difference was set at P<0.05.

Results

Low level of NR2F2-AS1 was closely associated with poor outcomes of GC patients

First, we investigated the expression of NR2F2-AS1 in GC tissues and cells. PCR assay indicated that NR2F2-AS1 was weakly expressed in GC specimens compared to corresponding samples (Fig. 1A). The level of NR2F2-AS1 in the advanced stage of GC was lower than that in the primary stage (Fig. 1B). Likewise, NR2F2-AS1 expression was down-regulated in GC cell lines (AGS, MGC-803, SGC-7901 and SNU-1) in contrast with normal gastric epitheliums GES-1 (Fig. 1C). More importantly, Kaplan-Meier analysis illuminated that the overall survival of patients with low NR2F2-AS1 level was worse than that of GC patients with high NR2F2-AS1 level (Fig. 1D). Moreover, the correlation between NR2F2-AS1 and clinical parameters of gastric cancer was shown in Table 1. Based on these results, we concluded that NR2F2-AS1 was lowly expressed in clinical samples and cell lines of GC. Additionally, down-regulation of NR2F2-AS1 predicted poor prognosis of GC patients.

Enhanced expression of NR2F2-AS1 restrained cell proliferation and promoted cell apoptosis in GC

In order to explore the biological role of NR2F2-

Table 1. the correlation between NR2F2-AS1 and clinical parameters of gastric cancer.

Clinical parameters	NR2F2-AS1		P value
	Low expression (n=18)	High expression (n=12)	
Age			0.073
>60	10	7	
≤60	8	5	
FIGO			0.039
I~II	8	8	
III~IV	10	4	
Tumor Size			0.016
>2 cm	12	3	
≤2 cm	6	9	
Gender			0.073
Men	8	7	
Women	10	5	
Lymph nodes status			0.058
Negative	6	6	
Positve	12	6	

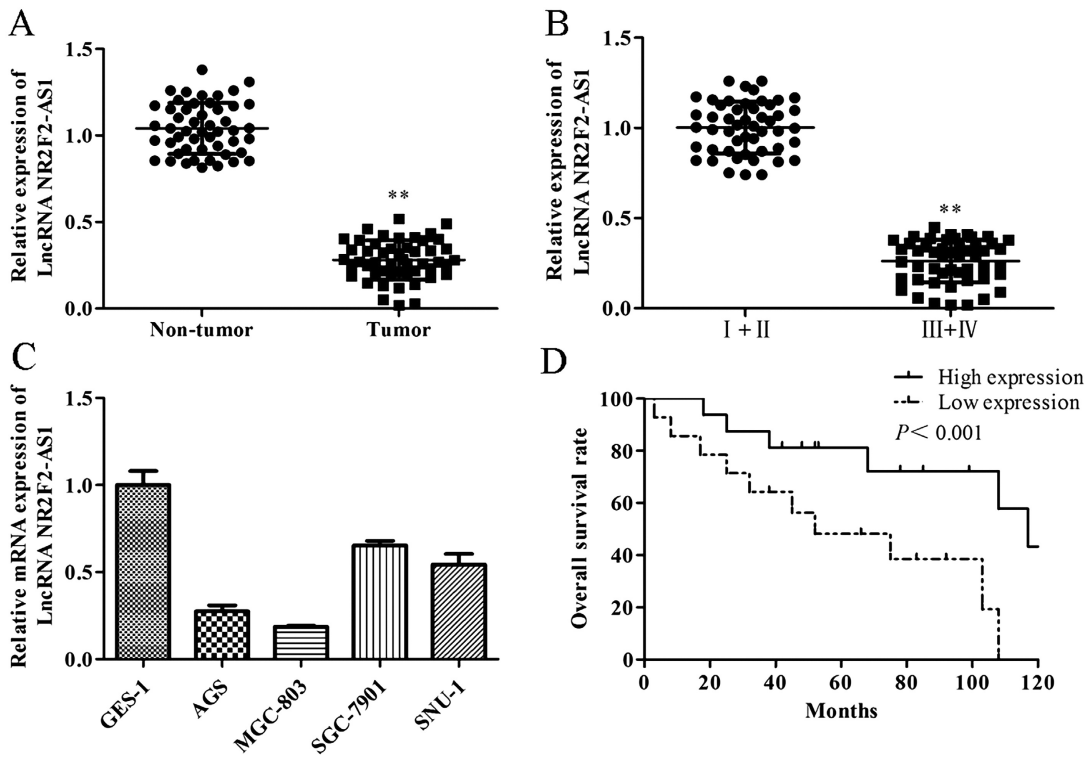

Fig. 1. Low level of NR2F2-AS1 was strongly associated with poor outcomes of GC patients. **A, B.** NR2F2-AS1 expression in GC tissues and normal samples as well as different grades was assessed by qRT-PCR. **C.** The qRT-PCR examined NR2F2-AS1 level in normal gastric epitheliums GES-1 and GC cells (AGS, MGC-803, SGC-7901 and SNU-1). **D.** The Kaplan-Meier analysis was adopted for plotting survival curves of GC patients. **P<0.01, ***P<0.001.

AS1 in GC, gain-of-function assays were implemented. We overexpressed NR2F2-AS1 in AGS and MGC-803 cells, and the efficacy of transfection was verified in PCR analysis (Fig. 2A). CCK-8 assay delineated that forced expression of NR2F2-AS1 inhibited the proliferative ability of AGS and MGC-803 cells (Fig. 2B). This result was further proven by a colony formation assay (Fig. 2C). Consistently, the TUNEL assay expounded that overexpression of NR2F2-AS1 led to the noteworthy augmentation of apoptotic cells (Fig. 2D). In general, ectopic expression of NR2F2-AS1 inhibited GC cell growth whereas it facilitated cell apoptosis.

NR2F2-AS1 overexpression impeded GC cell migration, invasion and EMT

Subsequently, we carried out transwell assays to determine the effects of NR2F2-AS1 on cell migration and invasion. It was displayed that up-regulation of NR2F2-AS1 contributed to the overt diminution of migrated cells (Fig. 3A). Similarly, NR2F2-AS1 overexpression alleviated the invasion of AGS and MGC-803 cells (Fig. 3B). We identified the role of NR2F2-AS1 in EMT considering that epithelial-to-mesenchymal transition (EMT) plays a pivotal part in the process of cell metastasis. Western blot elucidated that ectopic expression of NR2F2-AS1 resulted in the increase of E-cadherin level and the reduction of Vimentin expression (Fig. 3C). To sum up, NR2F2-AS1 suppressed cell migration, invasion and EMT in GC.

NR2F2-AS1 negatively regulated miR-320b via direct binding to it

Afterward, we intended to probe the molecular mechanism of NR2F2-AS1. With the aid of the starBase database, NR2F2-AS1 was uncovered to possess speculated binding sites with miR-320b (Fig. 4A). PCR assay showed that the expression of miR-320b was elevated in GC tissues and cell lines (Fig. 4B,C). RIP experiment illustrated that the enrichment of NR2F2-

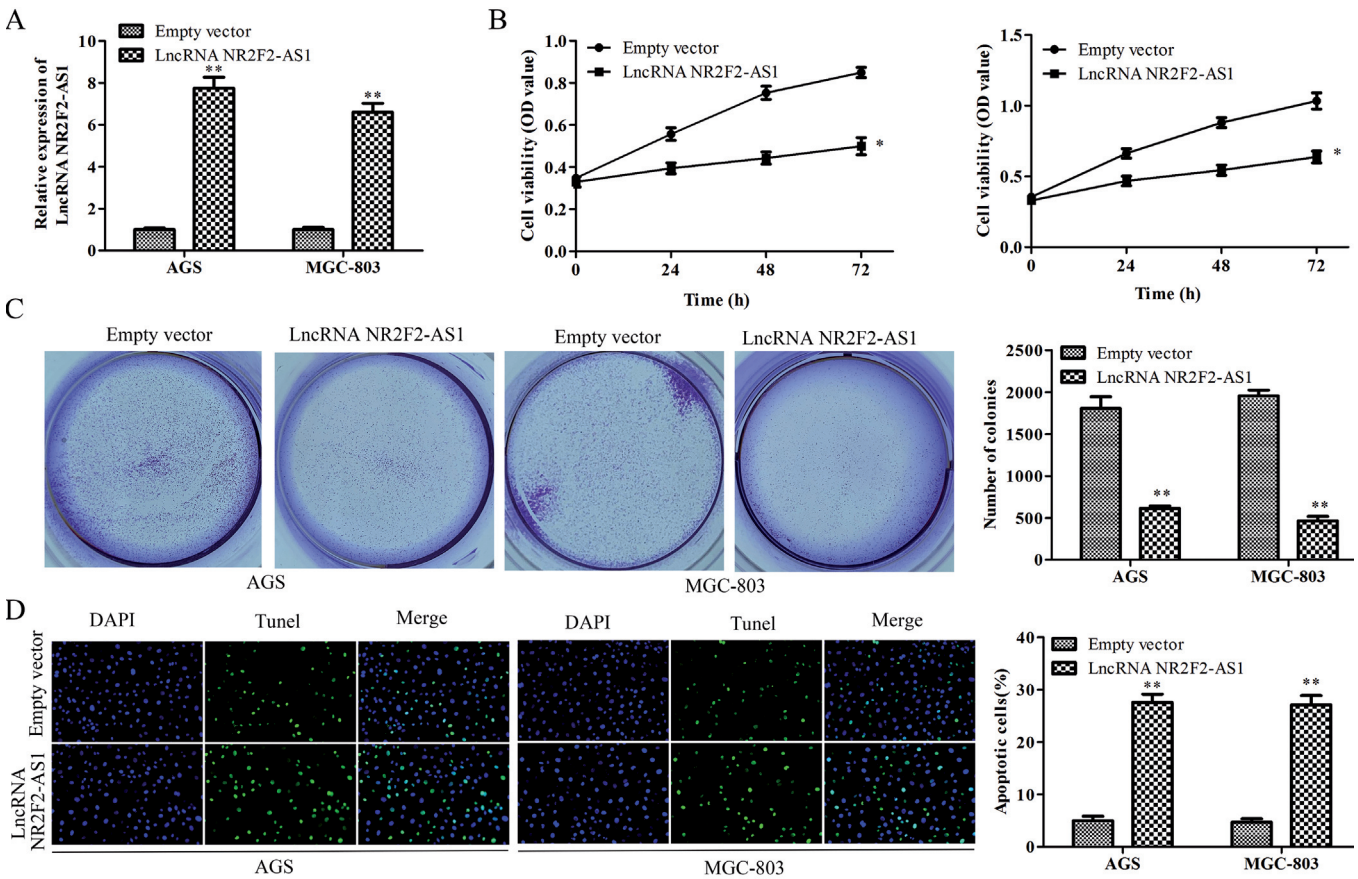

Fig. 2. Enhanced expression of NR2F2-AS1 restrained cell proliferation and promoted cell apoptosis in GC. **A.** The qRT-PCR analysis was applied to value efficacy of NR2F2-AS1 overexpression. **B, C.** After NR2F2-AS1 was up-regulated, the proliferation of AGS and MGC-803 cells was determined by CCK-8 assay and colony formation assay. **D.** TUNEL staining assay was implemented to estimate cell apoptosis after transfection. **P<0.01, ***P<0.001.

AS1 and miR-320b was observed in complex immune precipitated by Ago2 (Fig. 4D). Luciferase reporter assay revealed that luciferase activity of NR2F2-AS1-WT was lowered by miR-320b mimic, while that of NR2F2-AS1-Mut exhibited no obvious alteration, which further suggested that NR2F2-AS1 combined with miR-320b (Fig. 4E). In addition, up-regulation of NR2F2-AS1 led to the decline of miR-320b expression (Fig. 4F). The effectiveness of transfection for miR-320b was certified by PCR assay, and we demonstrated that overexpression of miR-320b significantly increased the miR-320b level (Fig. 4G). Considering the results above, Pearson's correlation analysis unveiled the negative relevance between NR2F2-AS1 expression and the level of miR-320b in GC samples (Fig. 4H). In short, these findings proved that miR-320b was sponged by NR2F2-AS1.

Suppression of miR-320b inhibited the development of GC

The expression of miR-320b was attenuated to perform loss-of-function experiments (Fig. 5A). It was viewed that repression of miR-320b reduced the viability of AGS and MGC-803 cells (Fig. 5B). Besides, the miR-320b inhibitor promoted cell apoptosis (Fig. 5C). Transwell assays expounded that miR-320b down-regulation inhibited the migratory and invasive capabilities of AGS and MGC-803 cells (Fig. 5D,E). Results of western bolt indicated that E-cadherin level was enhanced, while the expression of Vimentin was diminished when miR-320b was weakly expressed (Fig. 5F). On the whole, the described results provided strong evidence that miR-320b was a carcinogenic factor in GC.

NR2F2-AS1 sponged miR-320b to elevate the expression of PDCD4

PDCD4 was reported to act as the tumor suppressor of GC. Our findings confirmed that the expression of PDCD4 was overtly decreased in GC specimens and cells compared with normal ones (Fig. 6A,B). Considering that miR-320b binding sites were found in the 3'UTR of PDCD4 (Fig. 6C), we selected PDCD4 for an in-depth study. Our observations elucidated that forced expression of miR-320b decreased the luciferase activity of PDCD4-WT but it failed to regulate the luciferase activity of the mutated form (Fig. 6D). RNA pull down assay further validated the interaction of PDCD4 with miR-320b (Fig. 6E). Additionally, miR-320b overexpression brought about the reduced mRNA and protein expression of PDCD4 (Fig. 6F,G). The impacts of NR2F2-AS1 on the regulation of PDCD4 were evaluated by PCR assay and western blot. Up-regulation of NR2F2-AS1 heightened PDCD4 expression at mRNA and protein levels (Fig. 6H,I). Additionally, Pearson's correlation analysis disclosed that PDCD4 was negatively associated with miR-320b while being positively related to NR2F2-AS1 in tumor tissues (Fig. 6J). By and large, NR2F2-AS1 modulated PDCD4 level via competitively binding to miR-320b.

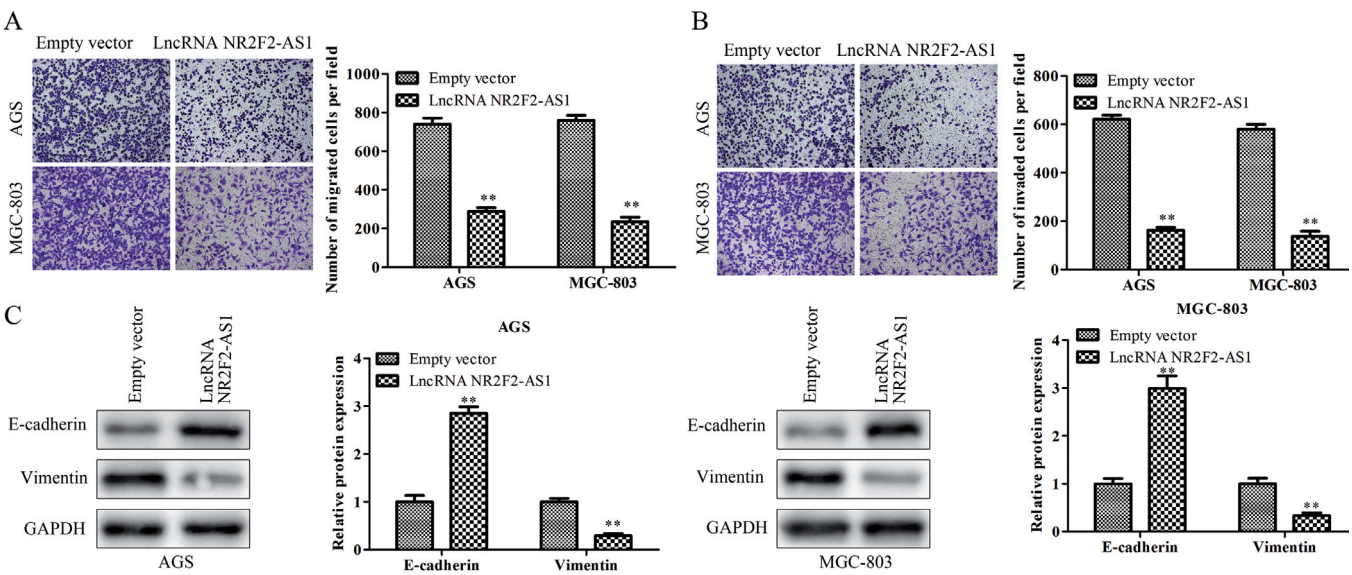

Fig. 3. NR2F2-AS1 overexpression impeded GC cell migration, invasion and EMT. **A, B.** Cell migration and invasion in AGS and MGC-803 cells were examined by transwell assays when NR2F2-AS1 was overexpressed. **C.** Western blot was conducted to detect the expression of EMT-related markers. ***P<0.001.

NR2F2-AS1 repressed the malignant phenotypes of GC cells via targeting miR-320b/PDCD4

Lastly, rescue assays were conducted to certify whether NR2F2-AS1 elicited its performance in GC through the miR-320b/PDCD4 axis. PCR assay expounded that PDCD4 was silenced after transfection (Fig. 7A). The CCK-8 and colony formation assays depicted that overexpression of miR-320b or depletion of PDCD4 boosted the weakened proliferative capacity caused by NR2F2-AS1 up-regulation (Fig. 7B,C). TUNEL assay demonstrated that cell apoptosis increased by overexpression of NR2F2-AS1 was recovered by miR-320b mimic or silencing of PDCD4 (Fig. 7D). Furthermore, the inhibitory influences of NR2F2-AS1 ectopic expression on cell migration and invasion were removed by up-regulation of miR-320b or PDCD4 knockdown (Fig. 7E). Concordantly, the blockade of the EMT process resulting from enhanced expression of NR2F2-AS1 was abrogated when miR-320b expression was fortified, or PDCD4 was down-regulated (Fig. 7F). Altogether, we corroborated that NR2F2-AS1 served as a cancer suppressor through sponging miR-320b/PDCD4 axis.

Discussion

Listed as the third most frequently occurring disease worldwide, GC threatens public health for its high morbidity and mortality (Ferlay et al., 2010). On account

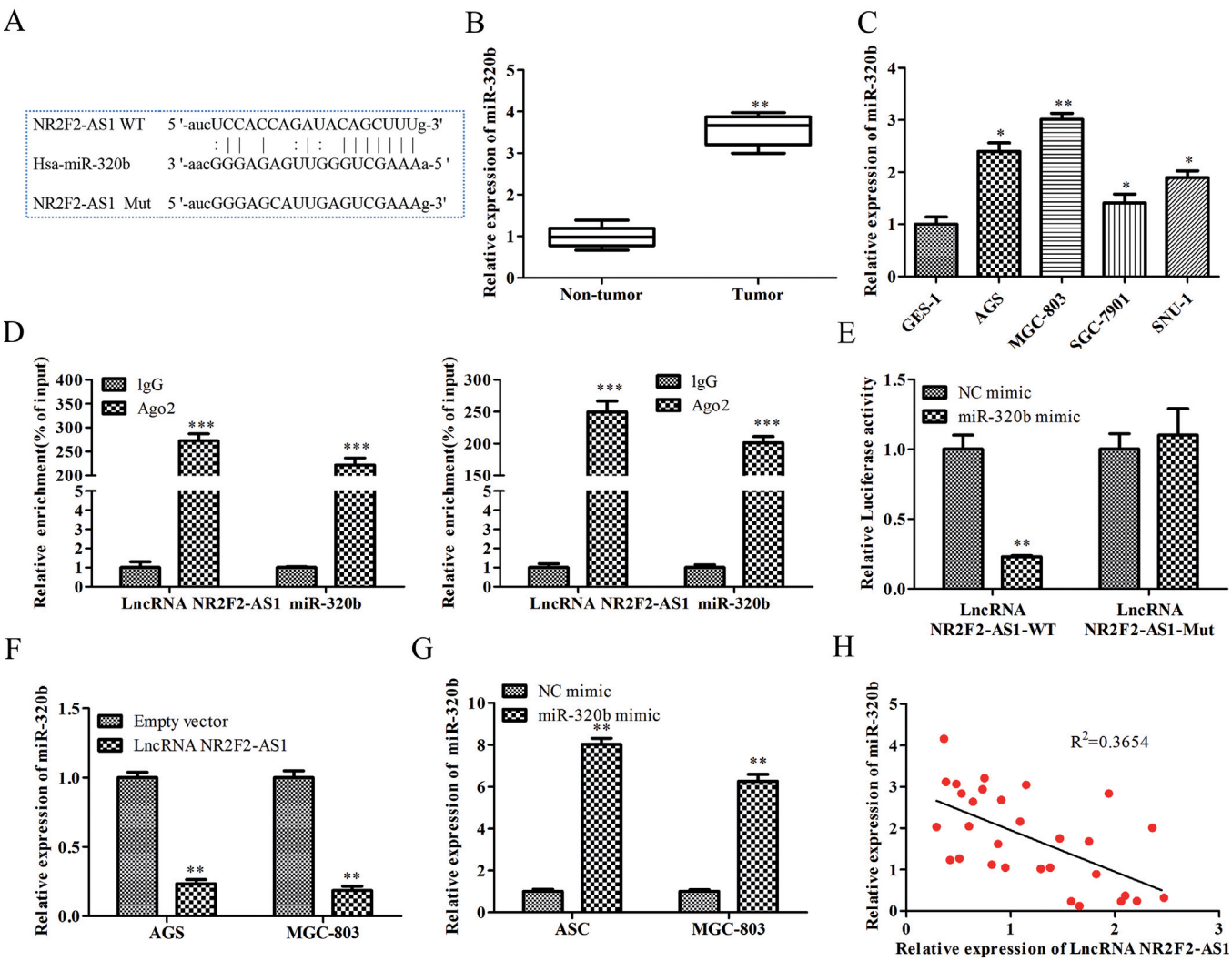

Fig. 4. NR2F2-AS1 negatively regulated miR-320b through directly binding to it. **A.** It was predicted by starBase that NR2F2-AS1 harbored miR-320b binding sites. **B, C.** The level of miR-320b in samples and cells of GC was tested by the qRT-PCR assay. **D, E.** To estimate the interaction between NR2F2-AS1 and miR-320b, RIP assay and luciferase reporter assay were implemented. **F, G.** NR2F2-AS1 and miR-320b expression as measured by qRT-PCR. **H.** The negative association between NR2F2-AS1 and miR-320b verified by Pearson's correlation analysis. **P<0.01, ***P<0.001.

of difficulties in the early detection, a majority of patients have reached the advanced stage at their first diagnosis (Eusebi et al., 2020). Although enormous advances in GC treatment have been attained, the prognosis of patients with GC remains dismal. Therefore, it is urgent to shed light on the latent mechanism of GC and discover efficient therapeutic targets for clinical application.

An increasing number of investigations highlight that lncRNAs are pivotal mediators in the occurrence and evolution of diverse malignant tumors (Bhan et al., 2017; Li et al., 2017; Deng et al., 2018). It has been testified that abnormal expression of lncRNAs is closely associated with carcinogenesis (Liu et al., 2014; Ma et al., 2016; Huang et al., 2017). NR2F2-AS1 has been corroborated to function as a cancer suppressor in several malignancies. For instance, NR2F2-AS1 accelerates cell proliferation by regulating the miR-4429/MBD1 axis in cervical cancer (Li et al., 2020a). NR2F2-AS1 up-regulates rac1 to increase cancer stemness in renal cell carcinoma (Chen et al., 2020). It is still unclear what the biological role and molecular mechanism of NR2F2-AS1are in the progression of GC. In the current study, we probed the expression level of NR2F2-AS1 in GC and then revealed that NR2F2-AS1 was prominently downregulated in GC tissues and cell lines compared to normal specimens and cells. Moreover, NR2F2-AS1 expression was lowered with

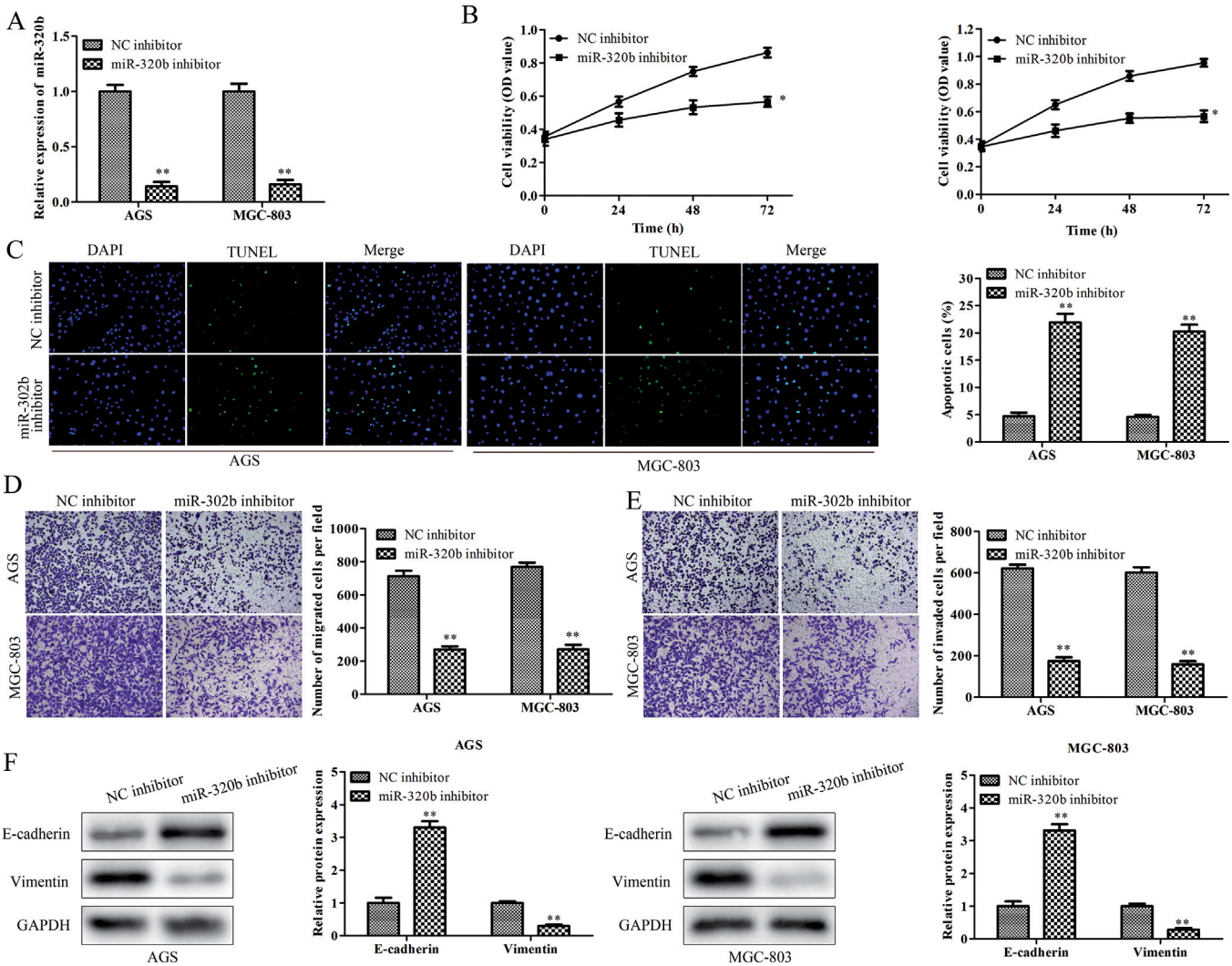

Fig. 5. Suppression of miR-320b inhibited the development of GC. **A.** The expression of miR-320b was assessed by qRT-PCR assay. **B.** The impacts of miR-320b in cell proliferation were evaluated by CCK-8 assay. **C.** TUNEL staining assay was used for analysis of cell apoptosis when miR-320b was repressed. **D, E.** Transwell assays were conducted to measure cell migration and invasion in AGS and MGC-803 cells transfected with NC inhibitor or miR-320b inhibitor. **F.** Western blot was applied to estimate the role of miR-320b in EMT. **P<0.01, ***P<0.001.

increased clinical classifications, and GC patients with low expression of NR2F2-AS1 had shorter overall survival time than those with high. Our results further expounded that overexpression of NR2F2-AS1 suppressed cell growth, migration, invasion and EMT.

As a type of small noncoding RNA, microRNAs (miRNAs) are validated to be vital regulators in affecting the expression of multiple genes at the post-transcriptional level via complementary base pairing with the 3'untranslated region (UTR) of target genes (Bartel, 2004). Mounting evidence has proven that miRNAs participate in various cellular processes and

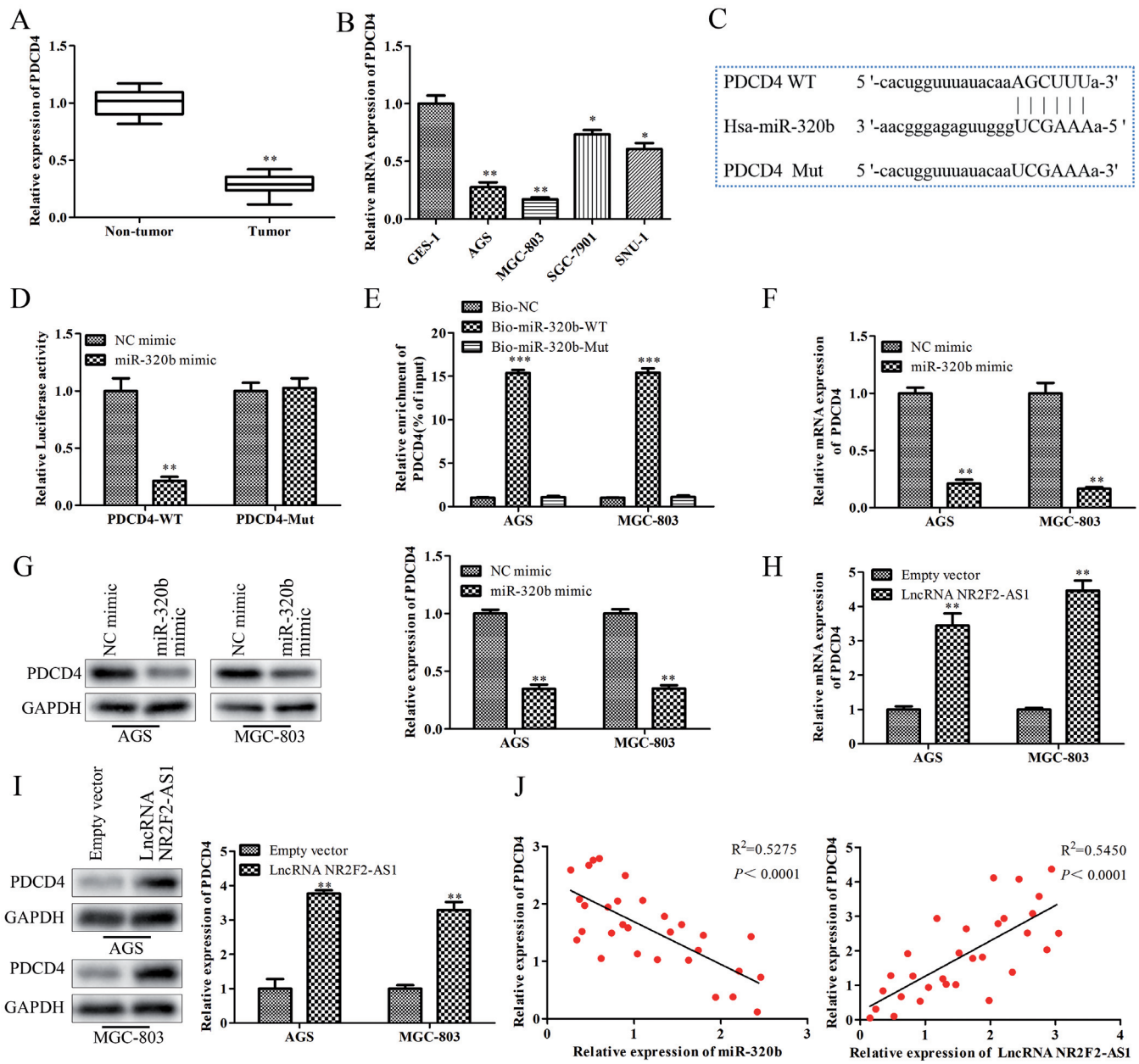

Fig. 6. NR2F2-AS1 sponged miR-320b to elevate the expression of PDCD4. **A, B.** Data of qRT-PCR assay of PDCD4 level in GC specimens and cells. **C.** StarBase uncovered that PDCD4 owned the putative binding sites with miR-320b. **D, E.** Luciferase reporter assay and RNA pull down demonstrated that miR-320b directly bound to PDCD4. **F-I.** The regulatory influences of NR2F2-AS1 and miR-320b on the mRNA and protein expression of PDCD4 were determined by qRT-PCR analysis and western blot. **J.** Pearson's correlation analysis was implemented to analyze the correlation among NR2F2-AS1, miR-320b and PDCD4. **P<0.01, ***P<0.001.

play critical roles in the pathogenesis of GC (Shin and Chu, 2014; Link and Kupcinskas, 2018). A myriad of studies illustrate that lncRNAs may act as ceRNAs so as to prevent the degradation of mRNAs mediated by miRNAs (Salmena et al., 2011; Pan et al., 2019). Recent literature demonstrates that lncRNA NR2F2-AS1 performs its function through the ceRNA regulatory network (Chen et al., 2019; Zhang et al., 2019). As a result, we presumed that NR2F2-AS1 might also serve as a ceRNA in GC tumorigenesis and its development. Through bioinformatics analysis, we found that NR2F2-AS1 harbored miR-320b binding sites. Considering the oncogenic role of miR-320b in hepatocellular carcinoma (Chen et al., 2020) and pancreatic cancer (Jingyang et al., 2021), we selected miR-320b for an in-depth study. Further experiments unveiled that miR-320b was sponged by NR2F2-AS1, and silencing of miR-320b alleviated the malignant behaviors of GC cells.

Programmed cell death 4 (PDCD4) is a novel tumor suppressor gene that has been reported to be involved in apoptosis, transformation, invasion and tumor progression. For example, tumorigenesis suppressor PDCD4 limits prooncogenic neuregulin-ErbB signaling to inhibit proliferation, migration and invasion in breast cancer (Montero and Pandiella, 2021). In addition, PDCD4 reduces the levels and activity of the p62-Nrf2 pathway through increasing Keap1, thereby inhibiting lung cancer tumorgenesis (Hwang et al., 2020). More importantly, tumor suppresser PDCD4 was proven to be a target of miR-320b. Mechanistically, our results clarified that NR2F2-AS1 inhibited the progression of GC through modulation of miR-320b/PDCD4.

On the whole, this work aimed to clarify the potential and regulatory mechanism of NR2F2-AS1 in GC. We unveiled that NR2F2-AS1 up-regulated PDCD4 to restrain the malignant phenotypes of GC cells by competing for miR-320b, which offered a deep comprehension on the molecular mechanism governing NR2F2-AS1 in GC and suggested that NR2F2-AS1 might represent an underlying potent target for the treatment of GC patients.

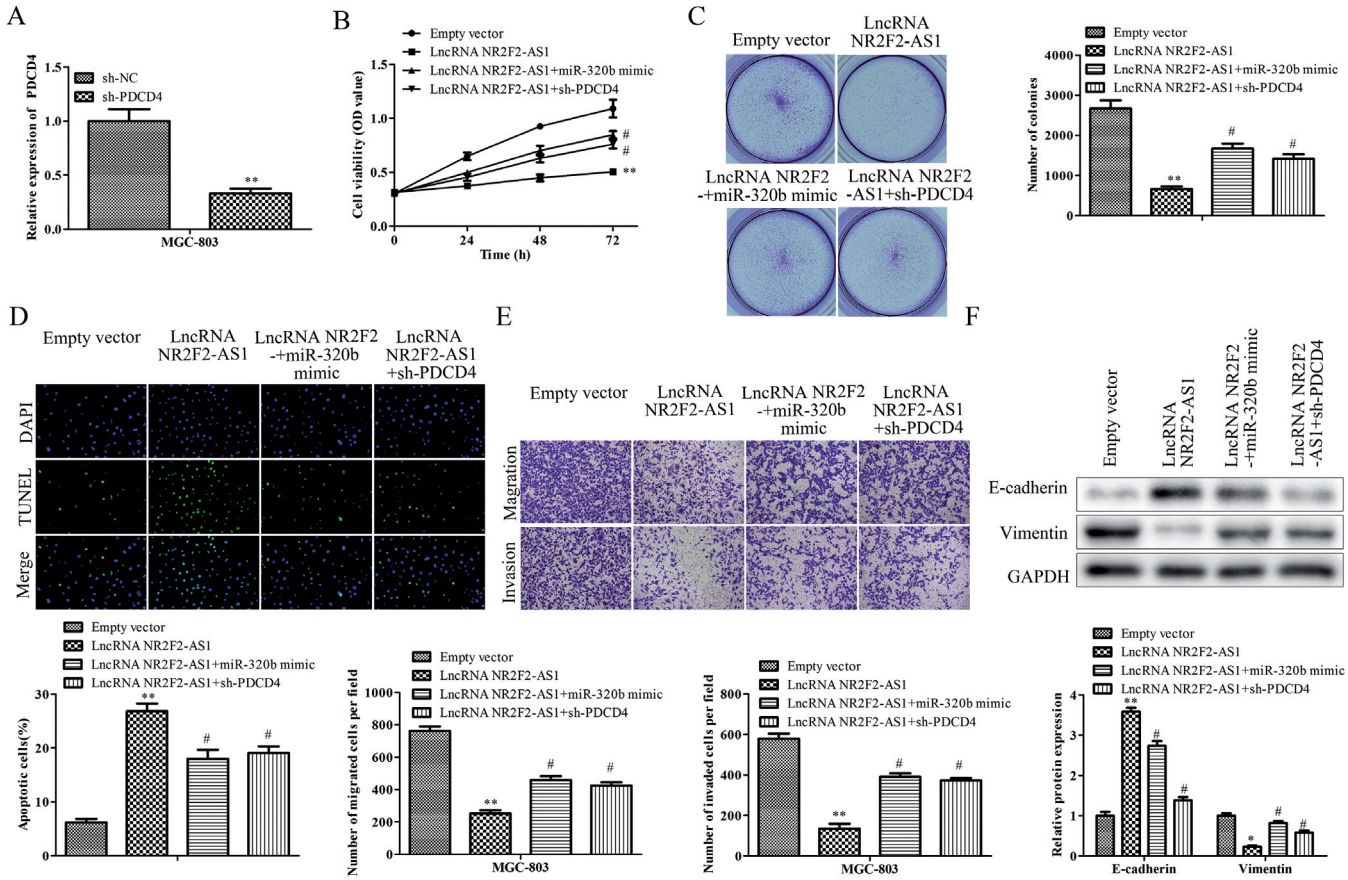

Fig. 7. NR2F2-AS1 repressed the malignant phenotypes of GC cells via targeting miR-320b/PDCD4. **A.** The effectiveness of transfection for PDCD4 was detected by qRT-PCR. **B, C.** The role of miR-320b/PDCD4 in cell proliferation was verified by CCK-8 assay and colony formation assay. **D.** Cell apoptosis was determined by TUNEL assay. **E.** Transwell assays were employed to assess the impacts of miR-320b and PDCD4 in cell migration and invasion. **F.** The expression of EMT-related proteins was examined by western blot. ***P<0.001. ##P<0.01, ###P<0.001.

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