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Novel circular RNA *circNF1* acts as a molecular sponge, promoting gastric cancer by absorbing miR-16

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Abstract

Circular RNAs (circRNAs) are a new class of RNA involved in multiple human malignancies. However, limited information exists regarding the involvement of circRNAs in gastric carcinoma (GC). Therefore, we sought to identify novel circRNAs, their functions and mechanisms in gastric carcinogenesis. We analyzed next-generation RNA sequencing data from GC tissues and cell lines, identifying 75,201 candidate circRNAs. Among these, we focused on one novel circRNA, *circNF1*, which was upregulated in GC tissues and cell lines. Loss- and gain-of-function studies demonstrated that *circNF1* significantly promotes cell proliferation. Furthermore, luciferase reporter assays showed that *circNF1* binds to miR-16, thereby derepressing its downstream target mRNAs, MAP7 and AKT3. Targeted silencing or overexpression of *circNF1* had no effect on levels of its linear RNA counterpart, *NF1*. Taken together, these results suggest that *circNF1* acts as a novel oncogenic circRNA in GC by functioning as a miR-16 sponge.

Key Words

- ▶ gastric carcinoma
- ▶ *circNF1*
- ▶ proliferation
- ▶ miR-16

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Introduction

Gastric carcinoma (GC) is the fourth most common cancer and third leading cause of cancer death worldwide (Fitzmaurice *et al.* 2015). Although research on GC has progressed substantially, its 5-year overall survival (OS) rate remains below 30% (Allemani *et al.* 2015); surgery remains the only potentially curative treatment (Van Cutsem *et al.* 2011). Thus, improvements in understanding are needed, including biomarkers for early diagnosis.

Recently, circRNAs have been identified as a new type of non-coding RNA. These transcripts comprise covalently closed circular RNA structures without 5'–3' polarity or a polyadenylated tail, features distinguishing them from linear RNAs. The first description of circRNAs in the cytoplasm of eukaryotic cells occurred in 1979 (Hsu & Coca-Prados 1979). However, due to limited detection technologies, circRNAs were considered errant

byproducts of RNA splicing and received little attention. With the advent of high-throughput RNA sequencing and improved bioinformatics, numerous circRNAs have now been identified (Jeck *et al.* 2013, Rybak-Wolf *et al.* 2015). Researchers agree that circRNAs represent a novel type of endogenous non-coding RNA, rather than errors. circRNAs are abundant in eukaryotic cells and exhibit evolutionary sequence conservation. Due to their resistance to exoribonuclease-mediated degradation, circRNAs are more stable than their linear counterparts (Jeck & Sharpless 2014). Based on the origin of their circular configurations, circRNAs can be classified as exonic, intronic or exonic-intronic. Exonic circRNAs occur principally in the cytoplasm and are the most-studied type to date (Memczak *et al.* 2013).

Increasing evidence indicates that numerous circRNAs are dysregulated and act as either oncogenic or tumor-suppressive factors in cancer initiation and progression. For example, circCCDC66 expression is elevated in colon cancer and is associated with a poor prognosis (Hsiao *et al.* 2017). Similarly, overexpression of circ-SMARCA5 promotes cell cycle progression and inhibits apoptosis in prostate adenocarcinoma (Kong *et al.* 2017). Because they often contain conserved miRNA-binding sites, many circRNAs function as endogenous miRNA sponges, reactivating miRNA downstream target genes by binding to and sequestering their corresponding miRNAs. One such circRNA, CDR1as, contains 73 conserved miRNA-7-binding sites, blocking miR-7-induced tumor suppression by antagonizing miR-7-mediated EGFR/RAF1/MAPK pathway suppression (Weng *et al.* 2017). In GC, several circRNAs have been implicated in carcinogenesis and progression (Zhang *et al.* 2017, Pan *et al.* 2018). However, functions and mechanisms of circRNAs in GC are still poorly understood.

In the current study, we analyzed next-generation sequencing data from GC tissues and cell lines for circRNAs, using the bioinformatic algorithm, circRNA_finder (Westholm *et al.* 2014). After data analysis and verification, *circNF1*, derived from exons 2–8 of linear, was chosen for subsequent experiments. *NF1*, a well-known tumor suppressor gene, is closely associated with neuroendocrine and endocrine tumors including neurofibromas (Gutmann *et al.* 2017), thyroid cancer (Uhlen *et al.* 2015) and carcinoid of the gastrointestinal tract (Swinburn *et al.* 1988). However, the existence of function of *circNF1* have not been previously studied or reported. We found that *circNF1* was upregulated in GC tissues and cells, and functional assays using interference and overexpression of *circNF1* revealed that *circNF1*

promotes cell proliferation. Finally, *circNF1* functioned as a miR-16 sponge, derepressing the miR-16 target genes *MAP7* and *AKT3*. We conclude that *circNF1* represents a novel potential biomarker and therapeutic target in GC patients.

Materials and methods

Bioinformatic identification of circRNAs from next-generation RNA sequencing (RNAseq) data

To identify expression levels of circRNAs in GC, an RNAseq dataset that we had generated (Huang *et al.* 2016) was studied using the circRNA_finder algorithm (Westholm *et al.* 2014). circRNA_finder aligns reads using Spliced Transcripts Alignment to a Reference (STAR) software (Dobin *et al.* 2013), extracting chimeric reads (reads that do not align continuously in a way that cannot be explained by regular splicing), finding chimeric reads consistent with backsplicing, and outputs these junctions with information regarding how many reads support each of them, as well as whether splice sites are canonical (Westholm *et al.* 2014). Canonical GT/AG splice sites are usually used as a major filter parameter in current circRNA identification strategies (Jeck & Sharpless 2014). circRNA_finder does not output which RNAseq reads support each junction, but rather only the number of reads supporting each junction. Next, we compared our results to circBase (www.circbase.org/) (Glazar *et al.* 2014), finally applying the COSMIC cancer census gene list (<https://cancer.sanger.ac.uk/census>) to prioritize cancer-related circular RNAs (Forbes *et al.* 2015). All circRNA junctions were stored in BED files; therefore, we used Bedtools to compare and merge datasets (<http://bedtools.readthedocs.io/en/latest/>) (Quinlan & Hall 2010).

Cell culture

Human GC cell lines MKN28, NCI-N87, AGS, KATOIII, RF1, RF48 and the normal gastric epithelial cell line HFE145 were cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco), incubated in a humidified 5% CO₂ atmosphere at 37°C.

Reverse transcription-polymerase chain reaction (RT-PCR) and Sanger sequencing

Total RNA was extracted with TRIzol reagent (Life technologies) and subjected to reverse transcription

with High Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific). Subsequently, divergent and convergent primers for each candidate circRNA were designed and used to amplify synthesized cDNA by TopTaq Master Mix kits (Qiagen). For linear RNA amplification, customary RT-PCR regular primers were applied. Finally, purified circRNA PCR products were subjected to Sanger sequencing to further confirm the correct circRNA backsplice junctions. All primers are contained in Supplementary Table 1 (see section on [supplementary data](#) given at the end of this article).

RNase R treatment

Total RNA (20 µg) from MKN28 or NCI-N87 was incubated at 37°C for 30 min with or without 2 U/µg of RNase R (Epicentre Biotechnologies). After purification with QIAprep Spin Miniprep kits (Qiagen), RNA was subjected to RT-PCR.

Patients and tissue specimens

Paired tumor and normal tissues from 23 patients obtained during surgical resections performed for clinical indications were enrolled in this study. This research was approved by the Institutional Review Board at the Johns Hopkins University School of Medicine. An informed consent form was signed by each patient. All age, gender, tumor size, differentiation degree and TNM stage data were exported from hospital records (Supplementary Table 4). All tissue samples were pathologically confirmed as GC. Tissues were studied for differential expression of *circNF1* and linear *NF1* mRNA by qRT-PCR.

Quantitative real-time PCR

After reverse transcription, cDNA was generated and amplified using SYBR PrimeScript or iQ Supermix kit (Bio-Rad) on an ABI 7900HT Fast Real-Time PCR system. Primers are listed in Supplementary Table 1. mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized against the threshold cycle (Ct) of GAPDH or β -actin. For detecting expression levels of miRNA, cDNA samples were amplified using TaqMan MicroRNA Assays (Applied Biosystems), and RNU6B was used as a reference gene.

Oligonucleotide transfection

2.0×10^5 cells/well of MKN28 and NCI-N87 GC cell lines were seeded in six-well plates before transfection.

siRNA (si-*circNF1*), targeted to interfere with the backsplice junction of *circNF1*, was designed and synthesized by Dharmacon (Lafayette, CO, USA). A negative nonspecific control siRNA (si-NC) was purchased from Dharmacon (Cat # D-001210-05). Subsequently, cells were transfected with a final concentration of 25 nM of either si-*circNF1* or si-NC using Lipofectamine RNAiMAX Reagent (Invitrogen) and incubated for 24 h for functional experiments.

For miRNA overexpression, miRNA mimics were synthesized by Dharmacon (Cat # C-300483-03-0002), and transfected into MKN28 cells using Lipofectamine RNAiMAX Reagent (Invitrogen) at a concentration of 60 nM.

Construction and transfection of *circNF1* overexpression vector

To generate a *circNF1* overexpression vector, the sequence of *circNF1* was first amplified using PCR. For subsequent cloning, a ClaI restriction enzyme site was added to the 5' end of one primer, while a SacII site was appended to the 5' end of the other primer. Primer sequences were forward, 5'-AATCGATGCTTCCAATAAAAACAGGACAG-3' and reverse, 5'-TCCGCGGCTTATTCATGTTGTTTTCATC-3'. Next, PCR products were cloned into pCR4-TOPO TA vector (Invitrogen). After verification by Sanger sequencing, verified correct plasmid was digested with ClaI and SacII enzymes (New England Biolabs, Inc.) and electrophoresed on 1% gels. After electrophoresis, bands were excised from gels and purified with NucleoSpin Gel and PCR Clean-up kits (Clontech). Isolated fragments were then ligated with pcDNA3.1 (+) circRNA mini vector, a generous gift of Jeremy Wilusz (Addgene plasmid # 60648) into the ClaI and SacII sites. pcDNA3.1 (+) circRNA mini vector contains several Alu elements, which are considered an important co-factor in forming circRNAs (Liang & Wilusz 2014). Following reconstitution of the circular vector, full-length *circNF1* was verified by Sanger sequencing.

After confirming the full-length sequence of *circNF1*, 2 µg of over-expression *circNF1* vector (*circNF1*) or empty vector (EV) were transfected into 2.0×10^5 cells/well of MKN28 (gastric cancer) and HFE145 (normal gastric epithelial) cell lines, respectively, using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. Backsplice junctions and efficiency of transfection were confirmed by Sanger sequencing and qRT-PCR, respectively.

Cell proliferation assays

Twenty-four hours after transfection, cells were harvested and reseeded onto 96-well plates at a density of

1000 cells/well in 100 μ L culture medium. 10 μ L of WST-1 reagent (Roche) were added to each well at the indicated time (day 0, 1, 3 and 5), and then incubated for 2 h at 37 °C in 5% CO₂ before measurement of absorbance. Optical density (OD) values were measured at 660 nm (background) and 440 nm (signal) on a microplate reader (Molecular Devices).

Colony formation assays

For colony formation assays, 1000 cells/well were seeded onto six-well plates and cultured for 12 days. Then, the plates were washed twice with PBS and stained with Diff-Quik Fixative, Diff-Quik Solution I and Diff-Quik Solution II (Dade Behring Inc, Newark, DE, USA) for 10 min each, in sequence at room temperature. Subsequently, colony numbers were counted and analyzed.

Scratch assays

Cells were transfected on six-well plates for 24 h and grown into monolayers. 200 μ L filter tips were used to create wound areas. At different time points (0, 24 and 48 h), scratch widths were photographed, and scratch healing rate was analyzed by Image J software.

Cell apoptosis assays

FITC Annexin V/Dead Cell Apoptosis Kits (Invitrogen) were used to measure apoptosis. Transfected cells were incubated for 24 h before harvesting. Following washing X 2 with cold PBS, collected cells were resuspended in 1 $\times$ annexin-binding buffer. Then, 5 μ L of FITC annexin V and 1 μ L of 100 μ g/mL PI working solution were added, respectively, into each cell suspension. After incubation for 15 min, 400 μ L of 1 $\times$ annexin-binding buffer were added before analyzing the stained cells by flow cytometry.

Dual-Glo luciferase assays

First, oligonucleotide pairs containing miR-16, miR-15b, miR-638, miR-515 and miR-194 target regions were ordered and annealed, respectively. Then, five pmirGLO Dual-Luciferase miRNA target expression vectors were constructed by inserting the respective annealed oligonucleotides into the pmirGLO vector (Promega) using PmeI and XbaI restriction enzymes (New England Biolabs, Inc). When fusion rate reached 70–90%, MKN28 cells were co-transfected with 80 ng of either *circNF1* overexpression plasmid (*circNF1*) or empty vector (EV),

along with 80 ng of either pmirGLO vector containing miRNA-binding sites or empty pmirGLO vector, into 1.0 $\times$ 10⁴ cells/well using Lipofectamine 2000 (Invitrogen). After 24 h, cells were analyzed for firefly and Renilla luciferase activity using the Dual-Glo Luciferase Assay Kit (Promega) and VICTOR2 fluorometry (Perkin Elmer).

Western blotting

The BCA protein assay procedure (Thermo Fisher Scientific) was conducted to determine protein concentration of the supernatant. Equal amounts of protein lysates were separated on 4–15% polyacrylamide gels (Bio-Rad) and transferred to 0.45 μ m supported nitrocellulose transfer membranes (Bio-Rad). After blocking with 5% skim milk at room temperature for 1 h, membranes were incubated with the following specific primary antibodies: AKT3 (1:2000, Cell Signaling), P-AKT (1:2000, Cell Signaling), MAP7 (1:500, Novus Biologicals) and anti- β -actin- peroxidase (1:30,000, Sigma-Aldrich) at 4 °C overnight. Subsequently, the membranes were washed three times with TBS containing 0.1% Tween 20 buffer (Sigma-Aldrich) and incubated with sheep anti-rabbit IgG secondary antibodies (1:2000, Cell Signaling) or horseradish peroxidase-labeled anti-mouse IgG (1:20,000, Invitrogen) for 1 h at 37 °C. Following washing three more times with TBST buffer, protein bands were detected by ECL Western blotting detection kits (GE Healthcare) according to the manufacturer's instructions. β -Actin antibody served as a control in these experiments.

Statistical analyses

All experiments were performed at least in triplicate. Discrepancies among group variables were analyzed by Student's paired or unpaired *t*-tests. Pearson correlation coefficient was used to compare correlations of *circNF1* and linear *NF1* with GC. Data were displayed as means $\pm$ s.e.m. of three experiments. All statistical analyses were conducted using SPSS 20.0, and statistical significance was defined as *P* < 0.05.

Results

Filtering of RNAseq data to select circRNAs

Figure 1 shows steps taken to extract and select circRNAs of interest from RNAseq data. First, we analyzed our RNAseq data (Huang *et al.* 2016) using the circRNA_finder algorithm (Westholm *et al.* 2014). These data contained

ten different samples: two matched normal (N)-tumor (T) tissues from two cancer patients; one normal tissue from a non-cancer patient; four unmatched GC tissues; two GC cell lines, NCI-N87 and MKN28 and one immortalized normal gastric epithelial cell line, HFE145 (Supplementary Table 2). Analysis yielded 75,201 circRNAs (chimeric reads) found in all samples, of which 14,963 circRNAs flanked a canonical splice site, an accepted filter parameter in current circRNA identification (Jeck & Sharpless 2014). Next, we compared our results with online circRNAs found in circBase: this comparison revealed 3643 circRNAs overlapping our circRNAs. Notably, unique chimeric reads occurred in each paired/unpaired tissue sample. In paired samples 2285 and 3245, overlapping circRNAs in cancer and adjacent tissues numbered 1205 and 1471, respectively (Table 1). Subsequently, to identify novel oncogenic circRNAs in GC, we filtered the entire list of circRNAs based on two prerequisites: circRNAs found in at least one tumor cell line and one tumor tissue sample and circRNAs found in COSMIC's list of 567 consensus cancer genes. Then, the generated circRNAs were ranked according to smallest BLAST E-value (i.e., the ORF most closely matching a database sequence). Finally, the highest 20 candidates were chosen for subsequent studies (Supplementary Table 3). For the current study, we randomly selected ten candidates for further investigation: *SETD2*, *FOXO3*, *ELK4*, *AFF4*, *MYH9*, *MSH2*, *CIC*, *FBXW7*, *NF1* and *ABL2*. The remaining ten circRNAs will be studied in future research.

Validation of selected circRNAs

To confirm backsplice junctions and spliced sequence lengths of the ten candidate circRNAs, both divergent and convergent primers were designed for RT-PCR. Results in NCI-N87 GC cells showed that with divergent primers, circRNAs derived from the genes *MSH2* and *ABL2* generated no PCR products of the expected size; circRNAs originating from the genes *ELK4* and *CIC* generated multiple amplicons; and the remaining candidates yielded single amplification products (Supplementary Fig. 1A). Three of the ten candidates (*MSH2*, *MYH9* and *NF1*) generated clear PCR products with convergent primers (Supplementary Fig. 1B). Thus, *MYH9* and *NF1* were the only two genes generating unique, correctly-sized amplicons from both divergent and convergent primers; these were named circMYH9 and circNF1 (Fig. 2A). Next, Sanger

Figure 1

Flowchart depicts work steps used to identify circRNA candidates of interest from RNAseq data.

sequencing of circMYH9 and circNF1 with divergent RT-PCR products was conducted to further validate and elucidate the backsplice junctions. After blasting sequence results in GenBank, we found that circMYH9 contained exons 39–40 of the antisense strand of *MYH9*, while circNF1 consisted of the sense strand of exons 2–8 of *NF1* (Fig. 2B). Finally, we compared expression levels of circMYH9 and circNF1 in 6 GC cell lines vs an immortalized normal gastric epithelial cell line, HFE145. circMYH9 was downregulated in GC cells compared with HFE145 cells (Fig. 2C), while circNF1 was upregulated in all GC cell lines (Fig. 2D). Since *NF1* is a well-known tumor suppressor gene (Xu *et al.* 1990), we hypothesized that circNF1 was likely to participate in gastric carcinogenesis. Therefore, circNF1 was chosen for further investigation.

In the UCSC genome browser, circNF1 (chr17:31155982–31182665), derived from the *NF1* locus on chromosome 17(q11.2), contains exons 2–8. After Sanger sequencing with convergent primers, we concluded that the full length of circNF1 was 828 bp, consistent with findings in the circBase database (hsa_circ_0042881). Because of its unique structure, circNF1 was treated with RNase R prior to reverse transcription to verify the lack of a 5' cap and 3' tail, and corresponding linear *NF1* transcript was used to demonstrate the efficacy of RNase R digestion (Fig. 2E). Then, we designed a traditional primer to amplify exons 1–9 of linear *NF1* to detect whether the biological mechanism of circNF1 is exon-skipping; however, no exon-skipped product was found (Fig. 2F), indicating that circNF1 was not a product of exon skipping.

Table 1 Numbers of circRNA candidates in different samples.

Sample	Total read counts	Unique counts	With canonical splice sites	In known DBs	% in DBs
2285T	91,655,404	10,436	2942	381	12.95
2285N	115,102,688	7229	1777	584	32.86
2285T-2285N		9231	2298	227	9.88
2285N-2285T		6103	1208	413	34.19
2285N $\cap$ 2285T		1205	644	154	23.91
3245T	199,449,428	9036	2065	508	24.60
3245N	164,431,037	7847	2480	590	23.79
3245T-3235N		7565	1320	328	24.85
3245N-3235T		6349	1703	402	23.61
3245N $\cap$ 3245T		1471	745	180	24.16
2284T	120,500,864	8503	2082	907	43.56
2286T	95,974,189	6689	1481	591	39.91
2289T	128,941,281	9150	2157	886	41.08
G008T	132,821,977	7529	1411	561	39.76
MKN28	103,919,937	7408	2196	908	41.35
NCI-N87	152,408,322	7764	2043	831	40.68
HFE145	117,020,409	6279	2088	883	42.29
2759NN	170,065,701	7884	2335	375	16.06
Merged	1,592,291,237	75,201	14,963	3643	24.35

Ten different samples were used to analyze novel circRNAs. 'Total read counts' contains overall chimeric reads (reads of all circRNA candidates ≥ 1); 'Unique counts' represents total number of novel circRNA candidates; 'DB' stands for circBase database; '% in DBs' equals the number of circRNAs in known DBs/circRNAs with canonical splice sites. In samples, T, tumor; N, normal; T-N, circRNAs present in tumor but not in normal tissues; N-T, circRNAs present in normal tissues but not in tumor tissues; N $\cap$ T, circRNAs present in both tumor and normal tissues.

Silencing of *circNF1* inhibits GC cell proliferation and migration but promotes apoptosis *in vitro*

To investigate the biological functions of *circNF1*, a specific siRNA targeting the backsplice junction of *circNF1* was designed (Fig. 3A) and transfected into MKN28 and NCI-N87 cells, the two GC lines that most highly expressed *circNF1* vs HFE145. Results of qRT-PCR showed that relative mRNA levels of *circNF1* were successfully and specifically knocked down, from a baseline of 1.00 to 0.06 and 0.08 in MKN28 and NCI-N87 cells, respectively ($P < 0.001$, Fig. 3B). Subsequently, WST-1 assays showed that knockdown of *circNF1* significantly decreased proliferation of MKN28 and NCI-N87 cells at day 5 ($P = 0.0079$, $P = 0.0212$, respectively; Fig. 3C and D). Similarly, colony formation ability was inhibited by transfection with si-*circNF1* vs transfection with si-NC in MKN28 and NCI-N87 cells ($P < 0.01$ and $P < 0.05$, respectively; Fig. 3E). Moreover, cell migration was also inhibited by transient transfection with specific anti-*circNF1* siRNA. Specifically, scratch assays revealed that silencing of *circNF1* caused a substantial reduction in cell migration at 48h in both cell lines ($P = 0.0196$, $P = 0.0188$; Fig. 3F and G). Next, flow cytometry assays were conducted to examine whether knockdown of *circNF1* induced programmed cell death or cell cycle arrest. Compared with si-NC, si-*circNF1* increased apoptosis to varying degrees of both cell lines ($P < 0.05$ and $P < 0.01$, Fig. 3H). However, no significant changes in cell cycle were observed (data not shown).

Overexpression of *circNF1* promotes cell proliferation *in vitro*

To further characterize the participation of *circNF1* in gastric carcinogenesis, we constructed a synthetic circRNA containing exons 2–8 of *NF1* using pcDNA3.1 (+) circRNA mini vector. HFE145, exhibiting the lowest native expression level of *circNF1*, along with MKN28, were chosen to transfect with either overexpression vector (*circNF1*) or empty vector (EV), after which *circNF1* expression levels were quantified by qRT-PCR. Results showed that this *circNF1* vector had been successfully constructed; relative to EV control, *circNF1* RNA levels were upregulated by 88.95- and 139.67-fold in MKN28 and HFE145 cells, respectively ($P < 0.001$, Fig. 4A). Next, we showed that overexpression of *circNF1* increased proliferation of both MKN28 and HFE145 cells at day 5 ($P = 0.0128$ and $P = 0.0181$, Fig. 4B and C). In addition, colony formation assays showed that overexpression of *circNF1* caused a significant increase in colony number, suggesting that *circNF1* promotes cell survival and proliferation ($P < 0.05$, Fig. 4D).

circNF1 acts as a miR-16 sponge

To explore molecular mechanisms exerted by *circNF1*, we performed online circRNA bioinformatics databases engines, including starBase v2.0 (<http://starbase.sysu.edu.cn/>).

Figure 2

Verification strategy of circRNA selection. (A) RT-PCR amplification products of *circMYH9* and *circNF1* using divergent ('Div') or convergent ('Con') primers. PCR products with divergent primers contain backsplice junction sites. Convergent primers' amplicons reflecting full-length *circMYH9* and *circNF1* are approximately 500 and 800 bp, respectively. (B) Head-to-tail splicing of *circMYH9* and *circNF1* was confirmed by Sanger sequencing of divergent PT-PCR products. The red arrows refer to backsplice junction sites. (C) Expression levels of *circMYH9* in gastric cancer cells vs the immortalized normal gastric epithelial cell line, HFE145. (D) Expression levels of *circNF1* in gastric cancer cells vs HFE145. (E) RNAs extracted from NCI-N87 and MKN28 gastric cancer cell lines were treated without or with (–/+) RNase R prior to reverse transcription, then amplified with *circNF1* or linear *NF1* primers. Only *circNF1* resists RNase R treatment. (F) RT-PCR with forward primer on exon 1 and reverse primer on exon 9 (to amplify exons 1–9 of linear *NF1*) and exon-skipped transcripts (exons 1/9, not detected) in NCI-N87 and MKN28 cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.00001$. A full-colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0478>.

edu.cn/starbase2/mirCircRNA.php), miRDB (<http://www.mirdb.org/>) and Circular RNA Interactome (<https://circinteractome.nia.nih.gov/>), which revealed that *circNF1* was potentially targeted by 63 different miRNAs, of which miR-194 and miR-515 appeared simultaneously in two of these databases. Next, our own previous miRNA microarray results generated from gastric cell line RNAs (Jin *et al.* 2011) were used to help identify the most likely carcinogenic miRNAs. In these data, 22 miRNAs were not detected in GC cells and baseline expression levels of 32 miRNAs were too low (cutoff level less than 0.5) to exert effects on cells, even if they became bound to *circNF1*. After this screening process, five putative miRNAs (miR-16, miR-15b, miR-638, miR-515 and miR-194) were determined to be likely candidates for further investigation. A flowchart describing this screening process of miRNAs targeted to *circNF1* is shown in Fig. 5A. Next, five luciferase plasmids, each containing

a respective miRNA-binding site, were constructed and subjected to luciferase assays (firefly luciferase activity/Renilla luciferase activity) to evaluate luciferase activity of each miRNA. Results showed that relative to the control luciferase plasmid, luciferase plasmids containing either miR-16 or miR-15b-binding sites significantly inhibited firefly luciferase activity (by at least 40%, Fig. 5B), indicating that endogenous miR-16 and miR-15b can be efficiently conjugated to luciferase reporter plasmids containing either miR-16 or miR-15b-binding sites. Subsequently, specific *circNF1* overexpression vector or empty vector was co-transfected with luciferase reporters containing miR-16- or miR-15b-binding sites into MKN28 gastric cancer cells. *circNF1* overexpression increased luciferase reporter activity of the luciferase plasmid containing the miR-16-binding site, suggesting that *circNF1* competitively inhibited endogenous miR-16 binding to luciferase plasmid by 'sponging'

Figure 3

Silencing of *circNF1* inhibits GC cell proliferation and migration but promotes apoptosis. (A) Schematic representation of backsplice junction and sequence of siRNA targeted to junction site of *circNF1*. (B) Results of qRT-PCR for *circNF1* in MKN28 and NCI-N87 cells treated with either si-NC or si-circNF1. (C) and (D) Results of WST-1 assay for proliferation after transfection with si-NC or si-circNF1. Inhibition of proliferation by knockdown of *circNF1* in MKN28 and NCI-N87 cells at day 5 ($P = 0.0079$ and $P = 0.0212$, respectively). (E) Representative images and quantification results of colony formation of knock-down *circNF1*. (F) Representative images of scratch assays in MKN28 and NCI-N87 cells transfected with control or *circNF1* siRNAs. (G) Line chart depicting scratch healing rates of MKN28 and NCI-N87 cells after silencing of *circNF1*. At hour 48t, wound closure in si-circNF1 group is significantly lower than in si-NC group in both MKN28 and NCI-N87 cells ($P = 0.0196$ and 0.0188 , respectively). (H) Representative images and quantification results of flow cytometry to evaluate apoptosis induction by *circNF1* in MKN28 and NCI-N87 cells transfected with si-NC or si-circNF1. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. A full-colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0478>.

miR-16 ($P < 0.05$, Fig. 5C). However, co-transfection of *circNF1* and the luciferase plasmid containing the miR-15b-binding site had no significant effect on luciferase activity (data not shown). Next, we explored expression levels of miR-16 after either knocking down or over-expressing *circNF1*. QRT-PCR confirmed that *circNF1* over-expression decreased miR-16 levels, while silencing of *circNF1* had the opposite effect in MKN28 gastric cancer cells ($P < 0.05$, Fig. 5D). We next investigated whether *circNF1* affected protein translation of downstream miR-16 target genes. In MKN28, over-expressed *circNF1* increased MAP7 protein levels, whereas translation levels of MAP7 were decreased by silencing of *circNF1* (Fig. 5E). Similarly, we found expression levels of AKT3 protein

were significantly increased by introduction of *circNF1*, but reduced after transfecting with si-circNF1. Moreover, AKT phosphorylation levels were also varied with either overexpressed or underexpressed *circNF1* (Fig. 5F). These data supported the hypothesis that *circNF1* functions as a miR-16 sponge and protects translational modification of MAP7 and AKT3 from attack by miR-16 (Spinetti *et al.* 2013, Yan *et al.* 2013). In addition, WST-1 assays demonstrated that a miR-16 mimic reduced the pro-proliferative effect of *circNF1* at day 5 ($P = 0.022$, Fig. 5G). Similar results occurred with colony formation assays ($P < 0.05$, Fig. 5H). Taken together, these findings indicated that *circNF1* bound to miR-16 and inhibited its activity.

Figure 4

Over-expression of *circNF1* promotes cell proliferation. (A) Results of qRT-PCR for *circNF1* in MKN28 and HFE145 cells without or with exogenous overexpression of *circNF1*. (B and C) WST-1 proliferation assay results in MKN28 and HFE145 cells transfected with either *circNF1* (*circNF1*) or empty vector (EV) at day 0, 1, 3 and 5. Proliferation increase at day 5 in cells treated by exogenous *circNF1* overexpression. ($P = 0.0128$ and $P = 0.0181$, respectively). (D) Representative images for colony formation after transfecting *circNF1* or EV. $*P < 0.05$; $***P < 0.001$. A full-colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0478>.

Expression of *circNF1* vs linear *NF1* mRNA in GC

Finally, we studied *circNF1* in 23 paired GC and normal tissues. Overall, expression *circNF1* was significantly elevated in GC tissues compared with adjacent normal tissues ($P = 0.0221$, Fig. 6A). Next, to understand whether the high levels of *circNF1* originated from its parental transcripts, we quantified levels of linear *NF1* mRNA in GC tissues. Surprisingly, unlike *circNF1*, expression levels of linear *NF1* were actually lower in GC tissues ($P = 0.0101$, Fig. 6B). We hypothesized that low expression of linear *NF1* could have resulted from its depletion to generate *circNF1*. Therefore, expression correlation of linear vs circular *NF1* in GC patients was analyzed by Pearson correlation coefficient. However, there was no significant negative correlation between linear *NF1* and *circNF1* ($r = -0.1348$, $P = 0.5397$, Fig. 6C). Similarly, consistent with the results in GC tissues, there was no correlation between linear and circular *NF1* transcript levels in GC cell lines (Supplementary Fig. 2). Moreover, neither targeted silencing nor overexpression of *circNF1* had any effects on linear *NF1* levels (Fig. 6D and E). Taken together, these results suggested that linear *NF1* and *circNF1* act independent roles in the development of GC.

Discussion

Recently, the involvement of circRNAs in human cancer has received intense attention. In the current study, we detected circRNAs enriched in GC by analyzing RNASeq data, identifying *circNF1* and *circMYH9* as *bona fide* exonic circRNAs. Moreover, we found that expression levels of *circNF1* were elevated in GC tissues as well as GC cells and that *circNF1* functioned oncogenically in

cellular functions including proliferation, migration and apoptosis. To our knowledge, this is the first report to explore the expression, function or regulation of *circNF1* in any human cancer.

Previous studies have demonstrated that circRNAs are abundant, diverse molecules expressed in a complex tissue- and cell-type-specific manner (Guo *et al.* 2014). To explore the expression and function of circRNAs in GC, we analyzed RNASeq data using bioinformatic algorithms, finding 75,201 distinct circRNA candidates. Notably, levels of circRNAs varied both inter- and intra-individually, suggesting that only a minority of circRNAs are shared between tumor samples in this particular tumor type and that differentially expressed circRNAs may exert potential carcinogenic functions in GC. Lin *et al.* found almost no overlapping circRNA candidates in 16 liver cancer samples. Similarly, only 0.5% of brain circRNA candidates were shared among 30 brain samples (Lin *et al.* 2017). One possibility underlying this apparent diversity may be that circRNA isoforms are commonly spliced into very specific pairs of exons (Salzman *et al.* 2013).

Both circRNAs and linear RNAs are generated from precursor mRNA. Unlike linear RNA, circRNAs are derived by canonical back-splicing. In addition, most reported circRNAs are generated exclusively via exonic circularization. Currently, proposed mechanisms of biogenesis of circRNAs include intron pairing, RNA-binding protein-driven circularization and exon-skipping (Ivanov *et al.* 2015, Kelly *et al.* 2015). In our study, we found that *circNF1* was derived from exons 2–8 of the *NF1* gene. Furthermore, with traditional primers for exons 1–9 of *NF1*, no exon-skipping product was found; thus, *circNF1* was apparently not derived from exon-skipping. It is worth noting that by using the GenBank and UCSC

Figure 5

circNF1 functions as a sponge of miR-16 in GC cells. (A) Schematic flowchart shows pipelines for screening miRNAs binding to *circNF1*. (B) Luciferase activity of luciferase vector containing each candidate miRNA binding site to detect endogenous miRNAs that were able to competitively bind to corresponding miRNA binding sequences in MKN28 cells. (C) Activity of luciferase plasmid ('Luc') containing or lacking a miR-16 binding site in MKN28 cells with vs without *circNF1* overexpression. (D) Results of qRT-PCR for miR-16 in MKN28 gastric cancer cells with either exogenous overexpression of *circNF1* or si-*circNF1*. (E) Representative images and quantitative analysis from Western blot of MAP7 protein with either over-expressed or under-expressed *circNF1* in MKN28 cells. (F) Representative images and quantitative analysis from Western blot of P-AKT and AKT3 in MKN28 cells treated with overexpression or silencing of *circNF1*, respectively. β -Actin antibody served as a control in these experiments. (G) WST-1 proliferation assay in cells transfected with *circNF1* or miR-16 as indicated. Proliferation decrease at day 5 in cells treated by combination of *circNF1* vector and miR-16 mimic relative to *circNF1* overexpression ($P = 0.022$). (H) Representative images for colony formation after either transfecting *circNF1* or miR-16 independently or co-transfecting *circNF1* and miR-16. $*P < 0.05$, $**P < 0.01$. A full-colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0478>.

genome browsers, we found abundant Alu repeats in the flanking regions of exons 2 and 8, which have been viewed as a necessary co-factor for the process of circularization (Ashwal-Fluss *et al.* 2014). In the formation of circHIPK3, long flanking introns with highly complementary Alu elements are indispensable to support circularization (Zheng *et al.* 2016). Therefore, we hypothesized that the formation of *circNF1* may be due in part to back-splicing with the help of intronic RNA pairing. However, the specific formation mechanism underlying *circNF1* deserves further exploration.

Accumulating research has indicated that circRNAs play important roles in carcinogenesis and cancer progression. In several studies, circRNAs function as tumor suppressors. For example, in bladder carcinoma, circHIPK3 was downregulated and correlated negatively

with cancer grade, invasion and lymph node metastasis (Li *et al.* 2017). Similarly, circLARP4 inhibited biological behaviors, including proliferation and invasion of GC cells, by 'sponging' miR-424, a known independent prognostic factor for tumor recurrence in GC (Zhang *et al.* 2017). In contrast, some studies showed that circRNAs may function as oncogenes. For example, circ-ABCB10 was significantly upregulated in breast cancer tissues, promoted proliferation and suppressed apoptosis. This same study also confirmed miR-1271 as a circABCB10-associated miRNA, which rescued the function of circABCB10 (Liang *et al.* 2017). Currently, the ability to function as a molecular sponge of native miRNA is the most well-documented function of circRNAs, protecting genes from attack by miRNAs. Our results demonstrated that *circNF1* was highly expressed in GC tissues as well as

Figure 6

Expression of *circNF1* and linear *NF1* in GC tissues and cell lines. (A and B) Results of qRT-PCR of *circNF1* and linear *NF1* in 23 GC tissues. β -Actin served as an internal control. (C) Pearson correlation coefficient analysis of correlation between expression levels of *circNF1* (x) and linear *NF1* (y) ($r = -0.1348$, $P = 0.5397$). (D) Results of qRT-PCR for linear *NF1* transcript levels in MKN28 and NCI-N87 cells transfected with either si-NC or si-*circNF1*. (E) Results of qRT-PCR for linear *NF1* transcript levels in MKN28 and HFE145 cells treated with either empty vector (EV) or circular *NF1* vector (*circNF1*). A full-colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0478>.

cell lines. We also found that exogenous overexpression of *circNF1* affected multiple tumor-associated cellular processes, including proliferation, migration and apoptosis, suggesting that *circNF1* functions as a gastric oncogene. In addition, online circRNA bioinformatics databases predicted that *circNF1* was potentially targeted by 63 different miRNAs, any of which could be involved in *circNF1* mechanisms. However, we found that expression levels of most of these 63 were too low to exert substantial effects mediated by *circNF1*. Therefore, we narrowed our exploration to five highly expressed miRNAs, focusing first on miR-16. The remaining 58 miRNAs and their involvement in other types of tumors expressing *circNF1* should prompt future studies to explore mechanisms of action of *circNF1*, for example, by constructing luciferase vectors.

We found that *circNF1* bound to miR-16 and affected translational levels of this miR's downstream target genes, MAP7 and AKT3, by 'sponging' miR-16. miR-16 has been reported as a tumor suppressor in multiple tumor types; its downregulation may enhance tumor cell proliferation, apoptosis and cell cycle progression by inhibiting miR-16's target activity on the 3'-UTR of oncogenes such as Bcl-2, Smad3 and Yap-1 (Cimmino *et al.* 2005, Kang *et al.* 2015, Zhang *et al.* 2018). Moreover, MAP7 (Yan *et al.* 2013) and AKT3 (Spinetti *et al.* 2013) has been previously validated as a direct target of miR-16 and decrease in the phosphorylated form of AKT pointed to dysregulation of the AKT-associated signaling pathway induced by miR-16 (Spinetti *et al.* 2013). Consistent with this previous observation, we observed increased translational levels of MAP7 and AKT3 as well as enhance phospho-AKT after sponging of miR-16 by *circNF1*.

In previous studies, linear *NF1*, a classic tumor suppressor gene, has been extensively studied; its product, neurofibromin, is an important negative regulator of Ras signaling (Xu *et al.* 1990). However, unlike linear *NF1*, our results suggest that *circNF1* functions as an oncogene. Moreover, there was no negative correlation between *circNF1* and linear *NF1* expression in GC tissues or cell lines, suggesting that regulation of *NF1* transcription and processing into circular RNA act independently during cancer development. *circNF1* was apparently not derived from exon-skipping, which may explain our finding that abnormal expression of *circNF1* did not affect linear *NF1* levels. Consistent with this result, circCCDC66 and circPVT1 are dysregulated in colon and gastric cancer, respectively, but expression levels of circular and linear RNAs of both CCDC66 and PVT1 are poorly correlated (Chen *et al.* 2017, Hsiao *et al.* 2017). However, a positive correlation was observed between circular and linear transcripts of circITCH and circFOXO3 (Huang *et al.* 2015, Yang *et al.* 2016). In cell lines, the current study showed that expression of *circNF1* has no effect on levels of linear *NF1* in either knockdown or exogenous overexpression experiments. This finding differs from previous studies showing that knockdown of exon-intron circRNAs, circEIF3J and circPAIP2, promoted EIF3J and PAIP2 transcription levels, respectively (Li *et al.* 2015). Taken together, our results suggest that *circNF1* and linear *NF1* represent independent transcripts in GC, and that *circNF1* is not generated from linear *NF1*. The relationship between circRNAs and their counterpart linear RNAs is worthy of further investigation. This relationship may provide additional insights into mechanisms of circRNA production.

In conclusion, based on RNASeq data bioinformatics analysis, we identified novel circRNAs expressed in GC. Among them, *circNF1* was highly expressed in GC. The biological functions of *circNF1* included stimulation of cell proliferation. Moreover, one molecular function of *circNF1* was shown to be regulation of the expression of MAP7 and AKT3 by functioning as a miR-16 molecular sponge. In summary, these findings support the belief that circRNAs are an important class of tumor biomarkers and potential therapeutic targets. Thus, studying molecular mechanisms of circRNAs should be a high priority for future researchers.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0478>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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