circCYFIP2 Acts as a Sponge of miR-1205 and Affects the Expression of Its Target Gene E2F1 to Regulate Gastric Cancer Metastasis

Jing Lin,1 Shasha Liao,2 E. Li,2 Zewa Liu,1 Ruihua Zheng,1 Xiaohua Wu,2 and Wanting Zeng3

1The First Affiliated Hospital of Shantou University Medical College, Shantou 515041, China; 2The First Affiliated Hospital of Shantou University Medical College, Longhu People’s Hospital Shantou, Shantou 515041, China; 3University College, London, UK

INTRODUCTION

Gastric cancer (GC) is responsible for a notable proportion of cancer morbidity and mortality worldwide.1 Although many mechanisms underlying the onset and progression of GC have been revealed over the past few years, delayed diagnosis and treatment are largely responsible for the high mortality rate among GC patients.2 Because there are limited therapeutic approaches for treating advanced GC, it is urgent that we search for novel biomarkers and prognostic indicators to reflect the disease status and develop more therapeutic targets for this lethal disease.3

Circular RNAs (circRNAs), a category of noncoding RNAs (ncRNAs), play a crucial role in the process of transcriptional and posttranscriptional gene expression.1 A large number of research studies have revealed that circRNAs perform critical functions in various physiological or pathological processes.1 circRNAs mediate gene expression by sponging microRNAs (miRNAs) or interacting with other molecules and then inhibit their function.4 miRNAs are a large class of short (~22 nt) ncRNAs that posttranscriptionally regulate gene expression through direct base pairing to target sites within miRNAs.5 circRNAs can affect miRNA activities by competing for miRNA-binding sites.6 However, the function of circRNAs as miRNA sponges has not been clearly elucidated in GC progression.

In this study, we aimed to investigate the role of circRNA in GC metastasis. We collected metastatic and non-metastatic GC tissues and analyzed their circRNA expression patterns. A novel circRNA circCYFIP2 was selected for further study. We assessed the relationship between circCYFIP2 expression and its diagnostic power and clinical significance in GC. Additionally, we investigated the role and underlying mechanism of circCYFIP2 in GC metastasis. Our findings highlight the important role of circCYFIP2-miR-1205-E2F1 axis in GC metastasis and its potential as a therapeutic target for GC.

RESULTS

circCYFIP2 Is Upregulated in GC Tissues and Correlates with the Progression and Poor Prognosis

To characterize metastasis-related circRNA transcripts, we conducted circRNA microarray analysis between 5 GC tissues with metastasis and 5 GC tissues without metastasis. We found a total of 863 differentially expressed circRNAs with a cut-off criteria of fold change >2.0 and p < 0.05, of which 487 were upregulated and 376 were downregulated in GC tissues with metastasis. The heatmap showed top 10 dysregulated circRNAs between GC tissues with metastasis and GC tissues without metastasis (Figure 1A). Expression levels of 10 circRNAs selected from the top 10 downregulated circRNAs were measured by quantitative real-time polymerase chain reaction in GC tissues with metastasis and GC tissues without metastasis (Figure 1B). Among the 863 differentially expressed circRNAs, 329,
including 183 upregulated ones and 146 downregulated ones, were verified as novel circRNAs; 534 circRNAs were identified beforehand and listed in the circRNA database (circBase, http://www.circbase.org; Figure 1C). The 863 identified circRNAs were divided into five different categories on the basis of the way they were produced. Exonic circRNAs consisting of the protein-encoding exons accounted for 74.04% (639/863), intronic circRNAs from intron lariats comprised 9.04% (78/863), sense overlapping circRNAs that originated from exon and other sequence circRNAs comprised 12.98% (112/863), and intergenic circRNAs composed of unannotated sequences of the gene and antisense circRNAs originating from antisense regions equally comprised 3.94% (34/863; Figure 1D). In the top 10 upregulated circRNAs, we found that circCYFIP2, also named hsa_circ_0003506 according to the annotation of circBase (http://www.circbase.org/), was the highest upregulated circRNA in GC tissues with metastasis, which was spliced from CYFIP2 gene located at chr5:156786012–156788606 and finally formed a sense-overlapping circular transcript of 366 nt.

To determine the level of circCYFIP2, we collected 68 pairs of fresh frozen GC tissues and matched normal tissues. The quantitative real-time PCR results showed that consistent with the results of GC cells, circCYFIP2 expression was higher in GC tissues than in normal tissues.
that GC patients with high circCYFIP2 expression had the significant correlation with clinicopathological features (Figure 1F). Furthermore, the Kaplan-Meier analysis showed that patients with M1 stage than these with M0 stage (Figure 1F), suggesting that circCYFIP2 is an oncogenic and metastasis-related circRNA. To investigate the functions of circCYFIP2 in GC cells, we designed and constructed small interfering RNAs (siRNAs) that targeted the junction sites of circCYFIP2 and overexpression plasmids of circCYFIP2 and transfected them into MKN-28 and SGC-7901 cells, respectively. circCYFIP2 expression was significantly silenced by si-circ-1, si-circ-2, and si-circ-3, while CYFIP2 mRNA did not change (Figure 2D). Similarly, circCYFIP2 was obviously overexpressed, and no significant change in CYFIP2 mRNA was observed (Figure 2E). Among the three si-RNAs, si-circ-1 had the highest knockdown efficiency in MKN-28 cells, so we chose the si-circ-1 in the following experiments. Inhibition of circCYFIP2 reduced the viability of MKN-28 cells (Figure 2F), whereas overexpression of circCYFIP2 promoted the proliferative ability of SGC-7901 cells (Figure 2G). In addition, knockdown of circCYFIP2 significantly decreased the number of cell colonies of MKN-28 cells, indicating that circCYFIP2 is highly stable in cytoplasm of GC cells.

circCYFIP2 Promotes Proliferation of GC Cells In Vitro
To test the functions of circCYFIP2 in GC cells, we designed three small interfering RNAs (siRNAs) that targeted the junction sites of circCYFIP2 and overexpression plasmids of circCYFIP2 and transfected them into MKN-28 and SGC-7901 cells, respectively. circCYFIP2 expression was significantly silenced by si-circ-1, si-circ-2, and si-circ-3, while CYFIP2 mRNA did not change (Figure 2D). Similarly, circCYFIP2 was obviously overexpressed, and no significant change in CYFIP2 mRNA was observed (Figure 2E). Among the three si-RNAs, si-circ-1 had the highest knockdown efficiency in MKN-28 cells, so we chose the si-circ-1 in the following experiments. Inhibition of circCYFIP2 reduced the viability of MKN-28 cells (Figure 2F), whereas overexpression of circCYFIP2 promoted the proliferative ability of SGC-7901 cells (Figure 2G). In addition, knockdown of circCYFIP2 significantly decreased the number of cell colonies of MKN-28 cells (Figure 2H) and promoted apoptosis (Figure 2I), whereas ectopic expression of circCYFIP2 in SGC-7901 cells led to an opposite result.

circCYFIP2 Enhances the Growth of Xenograft Tumors of GC Cells In Vivo
To investigate the functions of circCYFIP2 in vivo, we constructed the stable MKN-28 cells with sh-circCYFIP2 or sh-NC and SGC-7901 cells with circCYFIP2 or NC. The xenograft mouse model was established by subcutaneously injecting of GC cells. After 30 days, all mice were sacrificed and tumor samples were harvested. The volume (Figure 3A) and weight (Figure 3B) of tumors with knockdown of circCYFIP2 were markedly lower than those with control in MKN-28 cells, while the volume (Figure 3D) and weight (Figure 3E) of tumors with overexpression of circCYFIP2 were significantly higher than the control tumors of SGC-7901 cells. After harvesting the subcutaneous tumor tissues, immunohistochemistry (IHC) was performed. Results of

| Characteristics     | circCYFIP2 -21590444500 Low Expression High (n = 34) | p      |
|---------------------|------------------------------------------------------|--------|
| Age (years)         |                                                      |        |
| <60                 | 24                                                   | 20     | 0.446  |
| ≥ 60                | 10                                                   | 14     |        |
| Gender              |                                                      |        |
| Female              | 16                                                   | 13     | 0.624  |
| Male                | 18                                                   | 21     |        |
| Differentiation     |                                                      |        |
| Well                | 14                                                   | 9      | 0.305  |
| Moderate + poor     | 20                                                   | 25     |        |
| T stage             |                                                      |        |
| T1 + T2             | 25                                                   | 13     | 0.006  |
| T3 + T4             | 9                                                    | 21     |        |
| N stage             |                                                      |        |
| N0+ N1              | 17                                                   | 5      | 0.003  |
| N2+ N3              | 17                                                   | 29     |        |
| M stage             |                                                      |        |
| M0                  | 32                                                   | 24     | 0.023  |
| M1                  | 2                                                    | 10     |        |
| UICC stage          |                                                      |        |
| I                   | 26                                                   | 13     | 0.002  |
| II + III            | 8                                                    | 21     |        |
| Nerve invasion      |                                                      |        |
| Yes                 | 12                                                   | 16     | 0.460  |
| No                  | 22                                                   | 18     |        |
| Vessel invasion     |                                                      |        |
| Yes                 | 11                                                   | 14     | 0.615  |
| No                  | 23                                                   | 20     |        |

gastric tissues (Figure 1E). To further analyze the correlation between the level of circCYFIP2 with clinicopathological features and prognosis, we divided these samples into two groups, high circCYFIP2 group and low circCYFIP2 group, based on the median expression of circCYFIP2. As shown in Table 1, high level of circCYFIP2 was positively correlated with Union for International Cancer Control (UICC) stages, pathological T stages, lymphatic metastasis, distant metastasis, and grades. Also, the level of circCYFIP2 was higher in tissues with M1 stage than these with M0 stage (Figure 1F), suggesting that circCYFIP2 is an oncogenic and metastasis-related circRNA.
**circCYFIP2 Promotes Migration and Invasion of GC Cells In Vitro**

The mobility of GC cells was prominently decreased by downregulation of circCYFIP2, and the effect was also confirmed by the transwell migration and invasion assays in MKN-28 cells (Figures 4A and 4B). Nevertheless, overexpression of circCYFIP2 promoted the migration and invasion of SGC-7901 cells (Figures 4C and 4D). Taken together, these findings suggest that circCYFIP2 promotes the migration and invasion of GC in vitro.

**circCYFIP2 Acted as miR-1205 Sponge in GC Cells**

According to the hypothesis of competing endogenous RNA (ceRNA), circRNAs may promote the expression of target genes by sponging miRNAs. Since circCYFIP2 is located in the cytoplasm and exhibits high expression in GC cells, it is possible to act as a sponge for miR-1205.
stability, we speculated that circCYFIP2 could act as a miRNA sponge to increase target gene expression. We therefore analyzed the sequence of circCYFIP2 using miRanda, PITA, and RNAhybrid and identified five candidate miRNAs by overlapping the prediction results of the miRNA recognition elements in the circCYFIP2 sequence (miRNA-1205, miRNA-1184, miRNA-1827, miRNA-1256, and miRNA-1243; Figure 5A). It is well known that miRNAs usually silence gene expression by combining with the AGO2 protein and form the RNA-induced silencing complex (RISC). In the context of ceRNA mechanism, it might be a prevalent phenomenon that AGO2 could bind with both circRNAs and miRNAs. We therefore conducted an RNA immunoprecipitation (RIP) assay to pull down RNA transcripts that bind to AGO2 in MKN-28 and SGC-7901 cells. Indeed, endogenous circCYFIP2 was efficiently pulled down by anti-Ago2 (Figure 5B). To further detect whether circCYFIP2 could sponge miRNAs, we performed a miRNA pull-down assay using biotin-coupled miRNA mimics (miRNA-1205, miRNA-1184, miRNA-1827, miRNA-1256, and miRNA-1243). Interestingly, circCYFIP2 was only efficiently enriched by miR-1205, but not by the other four miRNAs (Figure 5C). To validate the interaction between circCYFIP2 and miR-1205, we obtained the binding sequence between miR-1205 and circCYFIP2 (Figure 5D) and conducted luciferase activity analysis. A subsequent luciferase reporter assay revealed that the luciferase intensity was reduced after the cotransfection of the wild-type (WT) luciferase reporter and miR-1205 mimics, while the mutated luciferase reporter exerted no such effect (Figure 5E, p < 0.01). In addition, RIP assay revealed that miR-1205 was efficiently pulled down by the anti-AGO2 antibody but not by the nonspecific anti-immunoglobulin G (IgG) antibody (Figure 5F). Furthermore, silencing of circCYFIP2 did not affect the expression of miR-1205, and transfection of miR-1205 mimics did not affect the expression of circCYFIP2 (Figures 5G and 5H), which indicated circCYFIP2 functions as a miRNA sponge without affecting the expression of sponged miRNAs.

**E2F1 Is a Direct Target of miR-1205**

To validate whether circCYFIP2 sponges miR-1205 and liberates the expression of its downstream target, we identified five target genes of miR-1205 by overlapping the prediction results of the four algorithms (miRanda, RNAhybrid, miRWalk, and TargetScan) prediction, and miR-1205 could target the 3′ UTRs of CRKL, MTA1, S100A8, E2F1, and BATF3 (Figure 6A). To further verify the downstream targets of circCYFIP2, we detected mRNA levels of five candidate target genes after silencing circCYFIP2, and we found that only E2F1 was downregulated (Figure 6B). To verify whether E2F1 was the direct target of miR-1205, we first performed the miRNA biotin pull-down assay. We found that miR-1205 could significantly enrich the 3′ UTR of E2F1 mRNA (Figure 6C). To verify whether the 3′ UTR of E2F1 mRNA was a target of miR-1205 in GC cells, we used a luciferase reporter gene assay. The WT 3′ UTR sequence or mutant (MUT) 3′ UTR sequence of E2F1 was cloned into a luciferase reporter vector. The luciferase activity was significantly inhibited by the miR-
1205 mimics in WT 3' UTR sequence-transfected cells. Conversely, the luciferase activity was not inhibited by the miR-1205 mimics in MUT 3' UTR sequence-transfected cells (Figures 6D and 6E). These results suggested that miR-1205 bind to the 3' UTR of E2F1 and directly downregulate E2F1 expression.

circCYFIP2 Promotes Cell Proliferation via the circCYFIP2-miR-1205-E2F1 Axis

To validate whether circCYFIP2 promotes cell proliferation via the circCYFIP2-miR-1205-E2F1 axis, we first confirmed that silencing circCYFIP2 decreased the mRNA and protein levels of E2F1 (Figures 7A and 7B), whereas overexpressing circCYFIP2 increased the mRNA and protein levels of E2F1 (Figures 7C and 7D). Then, we designed rescue experiments using miRNA-1205 inhibitors and mimics. At the protein level, miR-1205 partially reversed the effects of circCYFIP2 on E2F1 in GC cell (Figures 7A–7D). More importantly, as revealed by Cell Counting Kit-8 (CCK-8) assays, the miR-1205 mimics could partially rescue the proliferation-promoting effect induced by circCYFIP2 (Figure 7E). To determine the expression levels of E2F1 in GC, we analyzed the GC dataset from the The Cancer Genome Atlas (TCGA) database and found that the level of E2F1 was significantly upregulated in GC tissue compared with normal tissue (Figure 7F). However, Kaplan-Meier survival analysis from TCGA GC datasets suggested that high E2F1 expression in GC tissues is not significantly associated with OS (log-rank test, p = 0.25, Figure 7G) and disease-free survival (DFS; log-rank test, p = 0.22, Figure 7H). All of these data made us draw a conclusion that circCYFIP2 positively regulated E2F1 expression by interacting with miR-1205 in GC cells.

DISCUSSION

In this study, we investigated the expression profiles of circRNAs from 5 GC tissues with metastasis and 5 GC tissues without metastasis by microarray and focused on the expression level of circCYFIP2 in GC, and then elucidated the oncogenic role of circCYFIP2 and explored the underlying mechanism. Our study validated that circCYFIP2 can be considered as a promising biomarker for the diagnosis of GC. The expression of circCYFIP2 was remarkably higher in GC tissues and cell lines, and its upregulation was positively correlated with UICC stages, pathological T stages, lymphatic metastasis, distant metastasis, and grades in GC. Furthermore, we explored the function of circCYFIP2 in GC cells and found that overexpression or knockdown of circCYFIP2 significantly enhanced or reduced the invasive abilities of HCC cells. Specifically, we also showed mechanistically that circCYFIP2 promotes the progression of GC by acted as the sponge of miR-1205. Due to the stable loop structure and high abundance in the cytoplasm,
circCYFIP2 may be an efficient diagnostic and therapeutic target and a promising biomarker for prognosis in GC. First, one of the major findings of the present study is that a novel up-regulated circRNA, circCYFIP2, was identified in circRNA microarray analysis from 5 GC tissues with metastasis. Moreover, elevated expression of circCYFIP2 was positively correlated with UICC stages, pathological T stages, lymphatic metastasis, distant metastasis, and grades. Kaplan-Meier survival analysis demonstrated shorter OS in GC patients with high expression of circCYFIP2. ROC curves also suggested that the validated circCYFIP2 can serve as biomarkers for diagnosing GC.

Other than functioning as potential prognosis biomarkers for GC, circRNAs have also been shown to be associated with the carcinogenesis and aggressive behavior of GC. Here, in vitro functional assays revealed that gain-of-function experiments revealed that ectopic expression of circCYFIP2 promoted proliferation and inhibited apoptosis of GC cells. Loss-of-function experiments revealed that knockdown of circCYFIP2 inhibited proliferation and promoted apoptosis of GC cells. In addition, xenograft experiments showed that circCYFIP2 promoted GC xenograft growth in vivo. Moreover, our results showed that circCYFIP2 could significantly promote GC cell invasion ability in vitro, suggesting that circCYFIP2 might be a potential novel target for GC therapy.

Recently, increasing evidence has shown that circRNAs function as a sponge for miRNAs to affect tumorous biological process. It has recently been reported that circRNAs can act as miRNA sponges to negatively control miRNA. The majority of circRNAs can function
as sponges, via a mechanism of back-splicing, as they are enriched in miRNA binding sites. They can also competitively bind to miRNAs and decrease the activity of miRNAs. Our results further showed that circRNAs can serve as competitive endogenous RNAs and play an important role in GC development. Herein, using various assays, we found that circCYFIP2 promoted GC progression, mainly through interaction with miR-1205. Next, we verified that circFMN2 had an endogenous sponge-like effect on miR-1205 in GC. Furthermore, bioinformatics prediction and a luciferase reporter assay showed that circCYFIP2 and the E2F1 3′ UTR share identical miR-1205 response elements and might therefore bind competitively to miR-1205. Third, circCYFIP2 could bind directly to miR-1205 in an AGO2-dependent manner. Finally, circCYFIP2 regulates E2F1 expression by sponging miR-1205 because miR-1205 inhibition releases the inhibitory effect of circCYFIP2 deficiency on E2F1 expression.

miRNAs are a class of non-coding small RNAs of about 19–23 nucleotides in length, which are widely involved in various biological behaviors of cancer cells. miRNAs can post-transcriptionally reduce the levels of specific target protein coding gene expression by binding to the 3′ UTR of target mRNAs and resulting in translation inhibition or mRNA degradation. Studies showed that miRNAs were closely related to cell biological processes of GC. Recently, miR-1205 has been reported as a tumor suppressor that is significantly downregulated in multiple cancer types. In our study, E2F1 was predicted as the candidate target gene of miR-1205 by miRDB and Targetscan and was further testified by dual-luciferase reporter assay. Recently, a few studies have implicated that overexpression of E2F1 accelerates formation of various tumors. We found that E2F1 was upregulated in GC according to The Cancer Genome Atlas database (http://www.targe_tscan.org/vert_71/). However, high E2F1 expression in GC tissues is not significantly associated with worse OS and worse DFS.

In conclusion, our study reveals that circCYFIP2 is upregulated in GC tissues and that high circCYFIP2 expression is correlated with tumor metastasis and unfavorable prognosis of GC patients. Moreover, enhanced circCYFIP2 expression promoted GC cell growth and motility through sponging miR-1205 to upregulate E2F1. Consequently, circCYFIP2 may have considerable potential as a prognostic predictor and therapeutic target for GC. Our findings might provide new insight into GC development and provided a novel potential strategy for GC treatment.

MATERIALS AND METHODS

Tissue Collection
The trial was approved by the Research Ethics Committee of The First Affiliated Hospital of Shantou University Medical College, and written consent was obtained from all patients. 5 GC tissues with metastasis and 5 GC tissues without metastasis were used for microarray analysis. Furthermore, 68 paired GC samples were used for the validation of microarray analysis. All samples were collected from The First Affiliated Hospital of Shantou University Medical College, between January...
Cytological examination showed four patients with gastric adenocarcinoma, and graded according to the guidelines of the American Joint Committee on Cancer (AJCC).

**Microarray Analysis**
Samples (5 GC tissues with metastasis and 5 GC tissues without metastasis) were immediately frozen in liquid nitrogen after being obtained from surgical specimens. The sample preparation and microarray hybridization were performed based on the Arraystar’s standard protocols. After being digested with RNase R (Epigentek Technologies, Madison, WI, USA) to remove linear RNAs, circRNAs were amplified and transcribed into fluorescent circRNA utilizing Arraystar Super RNA Labeling Kit (Arraystar). Subsequently, the labeled circRNAs were hybridized onto the Arraystar Human circRNA Array (8 x 15 K, Arraystar), and then scanned by the Agilent Scanner G2505C (Agilent, CA, USA). circRNAs showing fold changes ≥2 and p values <0.05 were regarded as significantly differentially expressed.

**Bioinformatic Analysis**
Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Data processing were performed using the R software package. Differentially expressed circRNAs with statistical significance between two groups were identified through Volcano Plot filtering. Differentially expressed circRNAs between two samples were identified through Fold Change filtering. The circRNA/miRNA interaction was predicted with Arraystar’s homemade software based on miRanda, PITA, and RNAhybrid.

**RNA Extraction and Quantitative Real-Time PCR**
Total RNAs from cells and tissues were extracted using Trizol (Invitrogen, Carlsbad, CA, USA). Total RNAs from plasma were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. For statistical analysis, total RNAs were isolated from surgical specimens of GC tissues with metastasis (5 samples) and GC tissues without metastasis (5 samples) using TRIzol (Invitrogen, Carlsbad, CA, USA) and then evaluated by Volcano Plot filtering. The data and the corresponding clinical information of patients were collected from TCGA database (http://cancergenome.nih.gov/).

**TCGA Dataset Analysis**
The data and the corresponding clinical information of patients were collected from TCGA database (http://cancergenome.nih.gov/). We used the edgeR package of R packages to perform the difference analysis (http://www.bioconductor.org/packages/release/bioc/html/edgeR. html) and used the pheatmap package of R packages to perform the cluster analysis (https://cran.r-project.org/web/packages/pheatmap/index.html). Sva R package was used to remove the batch effect. Genes with adjusted p values <0.05 and absolute fold changes (FCs) >1.5 were considered differentially expressed genes. Kaplan-Meier survival curves were drawn to analyze the relationships between genes and overall survival in the survival package. The corresponding statistical analysis and graphics were performed in R software (version 3.3.2).

**Plasmid, siRNAs, and miRNA Mimic, Inhibitor, Transient Transfection, and Construction of Stable Cell Lines**
Plasmid-mediated circRNA overexpression and knockdown vector were obtained from Oebiotech (Shanghai, China), siRNAs targeting circRNA were obtained from GenePharma (Suzhou, China), miR-1205 mimic was ordered from RiboBio (Guangzhou, China), and the lentiviral expression vector of miR-1205 inhibitor and control plasmid were ordered from GeneCopoeia (Rockville, MD, USA). Cells were seeded in 6-well plates 24 h prior to miR-1205 mimic or inhibitor transfection with 50%–60% confluence and then were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. For stable transfections, we first established circRNA-0003506 and negative vector stable expressing cells using Puromycin selection methods.

**Actinomycin D and RNase R Treatment**
To block transcription, we added 2 mg/mL Actinomycin D or dimethyl sulfoxide (Sigma–Aldrich, St. Louis, MO, USA) as a negative control into the cell culture medium. For RNase R treatment, total RNA (2 μg) was incubated for 30 min at 37°C with or without 3 U/μg of RNase R (Epigenetics Technologies, Madison, WI, USA).
After treatment with Actinomycin D and RNase R, quantitative real-time PCR was performed to determine the expression levels of circ-CYFIP2 and CYFIP2 mRNA.

Isolating RNAs from Nucleus and Cytoplasmic Fractions
The nuclear and cytoplasmic fractions were isolated using PARIS Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. Briefly, cells were collected and lysed with cell fractionation buffer, followed by centrifugation to separate the nuclear and cytoplasmic fractions. The supernatant containing the cytoplasmic fraction was collected and transferred to a fresh RNase-free tube. The nuclear pellet was lysed with Cell Disruption Buffer. The cytoplasmic fraction and nuclear lysate were mixed with 2X Lysis/Binding Solution and then added with 100% ethanol. The sample mixture was drawn through a Filter Cartridge, followed by washing with Wash Solution and then added with 100% ethanol. The sample mixture was eluted with Elution Solution. U6 snRNA and GAPDH were employed as positive control for nuclear and cytoplasmic fractions, respectively.

CCK-8 Assay
Each group of cells in logarithmic phase was prepared into a single-cell suspension, and the cell density adjusted at 1,000 cells per well were seeded in a 96-well plate. Following that, six replicate wells were set in each group. On the second day, after the cells were attached, 10 μL of CCK-8 solution (Beyotime Biotechnology) was added to the sample, and a blank control well only containing the medium and CCK-8 solution was set. After incubating for 1 h, a microplate reader at a wavelength of 450 nm was used to determine and record the absorbance (OD) values of each well. Ultimately, the plate was measured at intervals of 24 h for 5 days.

Cell Apoptosis Flow Cytometry Analyses
At 48 h after transfection, transfected cells were harvested by trypsinization and resuspended in cold phosphate-buffered saline for analysis. The rate of cell apoptosis was detected using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s instructions.

Cell Wound Healing and Transwell Assays
For cell wound healing assay, the cells were first cultured to full confluence in 6-well plates. Subsequently, the cells were scratched with a 200 μL micropipette tip in the center of the well. Then, the cells were incubated with serum-free medium. Representative images were captured at 0 h and 24 h after injury. The width of wound healing was quantified and compared with baseline values. All experiments were repeated independently in triplicate.

Transwell experiment was carried out using transwell chamber (Millipore, Billerica, USA). In the migration assay, the transfected GC cells were centrifuged at 1,000 r/min for 3 min after trypsinization, and the cells were resuspended in serum-free medium and the density was adjusted to 1 × 10^5/mL. Then, 200 μL of the cell suspension was added to the upper compartment of the transwell chamber. In all, 700 μL of medium containing 10% FBS was added to the lower compartment. Then the cells were cultured for 24 h. Ultimately, the chamber was removed and the cells on the upper compartment were gently wiped with a cotton swab. Then, the cells passing through the membrane were fixed and stained with crystal violet solution for 30 min and washed twice with PBS. After they were dried, the cells were observed with a microscope, and five fields of view (×100) were randomly selected for counting. The mean value was considered as the number of migrated cells. In invasion assay, 50 μL Matrigel was diluted and coated on the upper compartment, and other experimental procedures were the same as the migration experiment.

Animal Experiments
To establish xenograft mouse models, we cloned the small hairpin RNA (shRNA) against circCYFIP2 (the same target with si-circ-1) and negative control into pLL3.7 vector, and we cloned the full-length cDNA of circCYFIP2 or a negative control into PLCDH-ciR vector, containing a front and back circular frame. Then, the stable cell lines with knockdown or overexpression of circCYFIP2 were constructed with MKN-28 or SGC-7901 cells. For in vivo tumorigenesis assay, 1.0 × 10^7 MKN-28 or SGC-7901 cells in 150 μL PBS were subcutaneously injected into left inguinal region of male BALB/c athymic nude mice (4 weeks old). Tumor volumes were calculated by the formula: tumor = (length × width^2)/2 and measured every 4 days. Finally, the mice were sacrificed, and the volume and weight of tumors were detected. The animal experiments complied strictly with the Animal Care guidelines of The First Affiliate Hospital, School of Medicine, Shantou University.

Immunohistochemistry
The expression of Ki67 in tumor tissues from nude mice was analyzed by immunohistochemical analysis. Briefly, the tissues were fixed with 4% formaldehyde for 24 h, embedded, and cut into 4-μm-thick sections. The sections were treated with 10 mmol/L sodium citrate buffer for 24 h, and then incubated with anti-Ki67 antibody (1:200 dilution) overnight at 4°C. The positive signaling was stained by using a mouse- and rabbit-specific horseradish peroxidase (HRP)/DAB (ABC) Detection IHC kit (Abcam Trading [Shanghai] Company, Shanghai, China), and counterstained with hematoxylin. The relative integral optical density (IOD) of positive signaling was obtained by ImageJ software.

RIP Assay
RIP assay was performed using an EZ-Magna RIP Kit (Millipore, Billerica, MA, USA) in accordance with the manufacturer’s instructions. Cells were lysed at 70%–80% confluence in RIP lysis buffer, and then incubated with magnetic beads conjugated with human anti-Ago2 antibody (Millipore) and normal mouse IgG control (Millipore) in RIP buffer. The RNAs in the immunoprecipitates were isolated with Trizol reagent and analyzed by quantitative real-time PCR.

Luciferase Reporter Assay
The sequence of circRNA containing the putative or mutated putative binding sites for miR-1205 was cloned into pmirGLO vector (Promega, Madison, WI, USA). PmirGLO-circCYFIP2-WT reporter and pmirGLO-circCYFIP2-MUT reporter were co-transfected into
cells with miR-1205 mimics and miR-NC. Lipofectamine 2000 was used for transfection. 48 h after transfection, luciferase reporter assay was performed using the dual-luciferase reporter assay system (Promega). The luciferase activity was normalized to Renilla luciferase activity.

**Western Blot Analysis**

For western blot analysis, cells were extracted using a protein extraction kit (Key Gene, KGP9100). Lipid proteins were added into 8%, 10%, 12%, or 15% gels, subjected to 120 V to promote migration, and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% BSA in TBST buffer and incubated with specific primary antibodies at 4°C overnight. The primary antibodies against E2F1 were obtained from Cell Signaling Technology (CST, Beverly, MA, USA). The next day, membranes were washed 3 times for 15 min in TBST and incubated with secondary antibodies for 2 h at room temperature. HRP substrate (WBKLO100, Millipore, USA) was used to detect the protein bands (Molecular Imager, ChemiDoc XRS+, Bio-Rad, USA), and the band intensities were quantified using Image-Pro Plus software (Mediacy, USA).

**Statistical Analysis**

Data were analyzed in GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Overall survival analysis was performed by Kaplan-Meier curves and log-rank test for significance. Student’s t test with two tails was used to assess the statistical significance in two groups and one-way ANOVA with post hoc Bonferroni test were used in three or more groups. Correlations were analyzed by Pearson correlation test. p <0.05 was considered statistically significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.05.007.

**AUTHOR CONTRIBUTIONS**

J.L. performed primers design and experiments. S.L. and E.L. contributed cell and animal experiments. Z.L. and R.Z. collected and classified the human tissue samples. W.Z. analyzed the data. W.Z. wrote the paper. All authors read and approved the final manuscript.

**CONFLICTS OF INTEREST**

The authors declare no competing interests.

**ACKNOWLEDGMENTS**

The datasets supporting the conclusions of this article are included within the article and Supplemental Information. Shantou Medical and Health Technology Project (No.180418184011332).

**REFERENCES**

1. Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J. Clin. 68, 394–424.
2. Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D., and Bray, F. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int. J. Cancer 136, E359–E386.
3. Zhao, T.T., Xu, H., Xu, H.M., Wang, Z.N., Xu, Y.Y., Song, Y.X., Yin, S.C., Liu, X.Y., and Miao, Z.F. (2018). The efficacy and safety of targeted therapy with or without chemotherapy in advanced gastric cancer treatment: a network meta-analysis of well-designed randomized controlled trials. Gastric Cancer 21, 361–371.
4. Qu, S., Yang, X., Li, X., Wang, J., Gao, Y., Shang, R., Sun, W., Dou, K., and Li, H. (2015). Circular RNA: A new star of noncoding RNAs. Cancer Lett. 365, 141–148.
5. Memczak, S., Papavasileiou, P., Peters, O., and Rajewsky, N. (2015). Identification and characterization of circular RNAs as a new class of putative biomarkers in human blood. PLoS ONE 10, e0141214.
6. Shang, Q., Yang, Z., Jia, R., and Ge, S. (2019). The novel roles of circRNAs in human cancer. Mol. Cancer 18, 6.
7. Salzman, J., Chen, R.E., Olsen, M.N., Wang, P.L., and Brown, P.O. (2013). Cell-type specific features of circular RNA expression. PLoS Genet. 9, e1003777.
8. Vo, J.N., Cieslik, M., Zhang, Y., Shukla, S., Xiao, L., Zhang, Y., Wu, Y.M., Dhanasekaran, S.M., Engelke, C.G., Cao, X., et al. (2019). The landscape of circular RNA in Cancer. Cell 176, 869–881.
9. Wang, N., Lu, K., Qu, H., Wang, H., Chen, Y., Shan, T., Ge, X., Wei, Y., Zhou, P., and Xia, J. (2019). CircBM33 regulates IL-6 to promote gastric cancer progression through targeting miR-149. Biomed. Pharmacother. 125, 109876.
10. Deng, G., Mou, T., He, J., Chen, D., Lv, D., Liu, H., Yu, J., Wang, S., and Li, G. (2020). Circular RNA circRHOBTB3 acts as a sponge for miR-654-3p inhibiting gastric cancer growth. J. Exp. Clin. Cancer Res. 39, 1.
11. Song, J., Wang, H.L., Song, K.H., Ding, Z.W., Wang, H.L., Ma, X.S., Lu, F.Z., Xiao, X.L., Wang, Y.W., Fei-Zou, and Jiang, J.Y. (2018). CircularRNA,100670 plays a critical role in intervertebral disc degeneration by functioning as a ceRNA. Exp. Mol. Med. 50, 94.
12. Su, H., Tao, T., Yang, Z., Kang, X., Zhang, X., Kang, D., Wu, S., and Li, C. (2019). Circular RNA CFFRC acts as the sponge of MicroRNA-107 to promote bladder carcinoma progression. Mol. Oncol. 18, 27.
13. Yang, R., Xing, L., Zheng, X., Sun, Y., Wang, X., and Chen, J. (2019). The circRNA circAGFG1 acts as a sponge of miR-195-5p to promote triple-negative breast cancer progression through regulating CCNE1 expression. Mol. Cancer 18, 4.
14. Ruan, Y., Li, Z., Shen, Y., Li, T., Zhang, H., and Guo, J. (2020). Functions of circular RNAs and their potential applications in gastric cancer. Expert Rev. Gastroenterol. Hepatol. 14, 85–92.
15. Yang, X., Zhu, J., Liu, Y., Chen, C., Liu, T., and Liu, J. (2019). Current Understanding of Circular RNAs in Gastric Cancer. Cancer Manag. Res. JJ, 10509–10521.
16. Liu, J., Wang, Y.H., Yoon, C., Huang, X.Y., Xu, Y., Xie, J.W., Wang, J.B., Lin, J.X., Chen, Q.Y., Cao, L.L., et al. (2020). Circular RNA circ-RanGAP1 regulates VEGFA expression by targeting miR-877-3p to facilitate gastric cancer invasion and metastasis. Cancer Lett. 471, 38–48.
17. Ambros, V. (2004). The functions of animal microRNAs. Nature 431, 350–355.
18. Nishibeppu, K., Komatsu, S., Imamura, T., Kiuchi, J., Kishimoto, T., Arita, T., Kosuga, T., Konishi, H., Kubota, T., Shiozaki, A., et al. (2020). Plasma microRNA profiles: identification of miR-1229-3p as a novel chemoresistant and prognostic biomarker in gastric cancer. Sci. Rep. 10, 3416.
19. Ozhawa, H., Kumagai, H., Miyaguchi, H., Miyato, H., Sakuma, Y., Horie, H., Hosoya, Y., Kawarai Lefor, A., Sata, N., and Kitayama, J. (2019). Exosomal microRNA in peritoneal fluid as a biomarker of peritoneal metastases from gastric cancer. Ann. Gastroenterol. Surg. 4, 84–93.
20. Li, P., Lin, X.J., Yang, Y., Yang, A.K., Di, J.M., Jiang, Q.W., Huang, J.R., Yuan, M.L., Xing, Z.H., Wei, M.N., et al. (2019). Reciprocal regulation of miR-1205 and E2F1 modulates progression of laryngeal squamous cell carcinoma. Cell Death Dis. 10, 916.
21. Yan, H., Chen, X., Li, Y., Fan, L., Tai, Y., Zhou, Y., Chen, Y., Qi, X., Huang, R., and Ren, J. (2019). MiR-1205 functions as a tumor suppressor by disconnecting the synergy between KRAS and MDM4/E2F1 in non-small cell lung cancer. Am. J. Cancer Res. 9, 312–329.
22. Endo, M., Tanaka, Y., Otsuka, M., and Minami, Y. (2020). E2F1-Ror2 signaling mediates coordinated transcriptional regulation to promote G1/S phase transition in bFGF-stimulated NIH/3T3 fibroblasts. FASEB J. 34, 3413–3428.
23. Zhao, M., Liu, Y., Chang, J., Qi, I., Liu, R., Hou, Y., Wang, Y., Zhang, X., Qiao, L., and Ren, L. (2019). ILF2 cooperates with E2F1 to maintain mitochondrial homeostasis and promote small cell lung cancer progression. Cancer Biol. Med. 16, 771–783.