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CircRNA circ-MYBL2 absorbs precursor miR-92b in the nucleus to suppress its role in enhancing gastric cancer cell proliferation

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Abstract

Background: MM-associated circular RNA (Circ-MYBL2), a circular RNA (circRNA), participates in cancer biology. However, its role in gastric cancer (GC) is unclear. In this study, circ-MYBL2 was predicted to interact with precursor microRNA-92b (miR-92b). We then analyzed the role of circ-MYBL2 in GC and explored its crosstalk with miR-92b.

Methods: In this research Circ-MYBL2 and miR-92b (mature and precursor) accumulation was determined using reverse transcription polymerase chain reaction (RT-qPCR). The involvement of circ-MYBL2 in the maturation of miR-92b was analyzed using overexpression assays. The subcellular location of circ-MYBL2 was determined using nuclear fractionation assay. The binding of precursor miR-92b to circ-MYBL2 was analyzed through RNA-RNA pulldown assay. The role of circ-MYBL2 and miR-92b in GC cell proliferation was studied with BrdU assay.

Results: We found that GC tissues exhibited increased mature miR-92b levels but decreased precursor miR-92b and circ-MYBL2 levels. Circ-MYBL2 was detected in both the nucleus and cytoplasm in GC cells, and it directly interacted with precursor miR-92b. Moreover, circ-MYBL2 overexpression increased precursor miR-92b expression and decreased mature miR-92b level. Furthermore, miR-92b (mature) increased GC cell proliferation, and circ-MYBL2 decreased GC cell proliferation and suppressed the effect of miR-92b on GC cell proliferation.

Conclusions: Circ-MYBL2 may absorb precursor miR-92b in the nucleus to suppress its role in promoting gastric cancer cell proliferation.

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Keywords: circ-MYBL2, gastric cancer, miR-92b, maturation

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Introduction

As a severe global health problem, gastric cancer (GC) affects more than 1 million people every year.[1, 2] Although the incidence of GC has gradually decreased, many GC patients are diagnosed at an advanced stage, making GC a leading cause of cancer deaths.[1-3] GC patients are usually treated with chemotherapy, radical surgery, radiation, and immunotherapy.[4, 5] Even for GC patients at the early stage or with localized tumors, recurrence can still occur in a considerable portion of patients after surgery with curative intent.[6, 7] Once recurrence occurs, patients' survival will be significantly affected.[8, 9] Therefore, safer approaches with higher efficiency are needed to further improve patients' survival.

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Altered gene expression and mutations in oncogenes and tumor suppressors are frequently observed in GC patients.[10, 11] Some molecular factors are considered potential targets to treat GC.[12] By regulating the expression of specific genes, certain cellular and/or molecular processes involved in cancers can be affected or reversed, thereby suppressing cancer development and progression.[13] Some molecular factors, such as Wnt5a and HER2, have been characterized as potential targets to treat GC.[12, 13] However, more effective targets are still needed to advance the development of targeted therapy for GC. Circular RNAs (circRNAs) are self-closed RNA transcripts with no or limited protein-coding ability but affect protein synthesis.[14-16] Altered expression of cricRNAs is frequently observed in human cancers, including GC.[17, 18] CircRNAs may regulate cancer cell behaviors, such as invasion, migration,

proliferation, drug resistance, and apoptosis by interacting with multiple signaling pathways to participate in cancer biology.[13, 14] Therefore, some circRNAs could be targeted to treat cancers, including GC. CircRNA MM-associated circular RNA (Circ-MYBL2) has recently been reported to participate in cancer biology.[19, 20] Circ-MYBL2 promotes the phosphorylation of MYBL2 in multiple myeloma, thereby suppressing the expression of a number of proliferation-related oncogenes, thereby inhibiting cell proliferation.[15] In cervical cancer, circ-MYBL2 sponges miR-361-3p to promote cell invasion and proliferation.[16] To date, the role of circ-MYBL2 in GC is unclear. Our preliminary sequencing analysis revealed the altered expression of circ-MYBL2 in GC (data not shown), suggesting its potential participation in GC. We predicted that circ-MYBL2 may interact with precursor miR-92b, an oncogenic miRNA in GC.[21] In GC, miR-92b activates DAB2IP-mediated PI3K/AKT signaling to promote tumor progression. Therefore, circ-MYBL2 may interact with precursor miR-92b to affect its maturation. We therefore analyzed the role of circ-MYBL2 in GC and its crosstalk with miR-92b.

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Methods

Paired tissue samples

Paired cancer and adjacent (with 3 cm to primary tumors) noncancerous tissues were donated by 62 GC patients who underwent D2 radical gastrectomy at Chengdu Wenjiang District People's Hospital from March 2018 to January 2021. Ethics approval was obtained. All tissue samples were obtained during the surgery, confirmed by 3 experienced pathologists and stored in liquid nitrogen. All patients signed written informed consent. All procedures were approved by the Ethics Committee of Chengdu Wenjiang District People's Hospital and operated in compliance with the standards set out in the Declaration of Helsinki and Laboratory Guidelines of Research in China. Procedures operated in this research were completed in keeping with the standards set out in the principles on ethical animal research outlined in the National Institutes of Health Laboratory Animal Care and Use Guidelines. Patients were excluded if they had other gastric diseases (except the known risk factors for GC) and other severe diseases and treated with other anti-cancer therapy within 6 months prior to admission. Table 1 lists the patients' clinicopathological data.

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GC cell lines and cell culture

Two GC cell lines MKN-28 and BGC-823 and GES-1 (normal) were obtained from ATCC (USA) and used in *in vitro* cell experiments. MKN-28 and BGC-823 cells were cultivated in RPMI 1640 medium. Dulbecco's Modified Eagle Medium

(DMEM) was used to cultivate GES-1 cells. Fetal bovine serum (10%), penicillin (100 U/ml) and streptomycin (100 μg) were added into medium. Cells were cultivated in a 5% CO_2 incubator at 37°C. Cells collected at 70-80% confluent were collected for the subsequent experiments.

Transient cell transfections

The short interfering RNA (siRNA) for circ-MYBL2 were generated by Fulengen (Guangzhou, China). Circ-MYBL2 and miR-92b were overexpressed in both MKN-28 and BGC-823 cells by transiently transfecting 8 μg pcDNA3.1-circ-MYBL2 expression vector or 20 nM mimic of miR-92b (Invitrogen) into 10^7 cells, respectively.

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Lipofectamine 2000 (Thermo Fisher Scientific) was applied to perform all transfections. To achieve transfection, cells were incubated with transfection mixture (Lipofectamine 2000 + vector or miRNA) for 6h, followed by washing with fresh medium. Their overexpression was checked every 24h until 72h. In addition, empty vector or negative control (NC) miRNA were also transfected into both cell lines as NC using the same system. Cells without transfection were used as the control.

RNA isolation and processes

Total RNAs from all samples were isolated using Quick-RNA Microprep (R1050, ZYMO) and eluted in RNase-free water. After DNase I digestion to remove genomic DNA, RNA concentrations were measured using Epoch spectrophotometry, and RNA integrity was analyzed with the 2100 Bioanalyzer system. RNAs with a RIN higher

than 1.8 were obtained. Otherwise, RNA isolation and processes were repeated.

Reverse transcription polymerase chain reaction (RT-qPCR) Using QuantiTect Reverse Transcription Kit (QIAGEN) at the following thermal conditions: 5 min at 23°C, 30 min at 55°C, and 10 min at 85°C, cDNA samples were prepared. The obtained cDNA samples were used as templates in qPCR to analyze circ-MYBL2 a miR-92b expression with 18S rRNA internal control. The primer sequences were 5'-TGGACACAGGAGGCTTCTTGAG-3' (forward) and 5'-ATCGGTACAGATCTGGACGCAG-3' (reverse) for circ-MYBL2; 5'-CTACCACATCCAAGGAAGCA-3' (forward) and 5'-TTTTTCGTC ACTACCTCCCCG-3' (reverse) for 18S rRNA; 5'-GCTTCGGCAGCACATATACTAAAAT-3' (forward) and 5'-CGCTTCACGAATTTGCGTGTCAT-3' (reverse) for U6; 5'-GCCCCGGGCGGGCGGGAG-3' (forward) and 5'-CGGGGGGGCCGGAGG-3' (reverse) for precursor miR-92b; 5'-GTCTCCTCTGACTTCAACAGC-3' (forward) and 5'-CCACCCTGTTGCTGTAGCCAA-3' (reverse) for GAPDH; and 5'-TATTGCACTC GTCCCGGCC-3' (forward) and universal reverse primer for mature miR-92b. The primers position information for various forms miR-92b was shown in TableS2. The method of $2^{-\Delta\Delta Ct}$ was used to normalize Ct values.

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RNA-pull down assay with biotin-ligated RNAs

In vitro transcripts of precursor miR-92b (miR-92b (pre)) and NC were prepared.

Both RNAs were labeled with biotin using Biotin RNA Labeling Mix (Sigma Aldrich). MKN-28 and BGC-823 cells were transfected with either biotin-labeled miR-92b (pre) (Bio-miR-92b (pre)) or NC miRNA (bio-NC) using the methods mentioned above. Following that, cells were cultured for 48h and lysed. RNA complexes in both cell lysates were pulled down with streptavidin-coupled agarose beads (ThermoFisher), extracted using Quick-RNA Microprep (R1050, ZYMO), and subjected to RT-qPCR to analyze circ-MYBL2 expression.

Nuclear and cytoplasmic fraction assay

The nuclear (N) and cytoplasmic (C) fractions of MKN-28 and BGC-823 cells were prepared using the PARIS kit (AM1921, ThermoFisher Scientific). After centrifugation at 2500g for 15 min, the supernatant was collected as the C fraction and the pellet was collected as the N fraction. After that, RNA was isolated from both fractions, prepared as cDNA samples, and subjected to PCRs using Taq DNA polymerase (NEB) to amplify circ-MYBL2.

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BrdU incorporation assay

In this study, BrdU incorporation was measured to reflect DNA synthesis, which directly reflects cell proliferation. Cells were collected at 48h after transfections and seeded onto a 96-well cell culture plate with 5000 cells per well and incubated with 10 μM BrdU for 24h. After incubating with peroxidase-coupled anti-BrdU-antibody (Sigma-Aldrich) and tetramethylbenzidine, OD values at 450 nm were measured. The

OD values of cells treated with BrdU but not BrdU antibody were considered background.

In vivo tumor xenograft assay

Lentivirus particles expressing circ-MYBL2 and miR-92b (Vigenebio, Jinan, China) were transfected into MKN-28 cells following manufacturer's instruction. Experimental procedures were approved by Institutional Review Board of the Chengdu Wenjiang District People's Hospital. Stable MKN-28 transfection cells (10^7) were harvested and re-suspended in 50% Matrigel. The mixture was injected into nude mice (male, 4-week-old, Vital River Laboratories, Beijing, China). Tumors were harvested 4 weeks after infection and photographed. Tumor volume was calculated using the formula: volume = (length × width^2)/2.

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Northern blot analysis

Northern blotting analysis was performed using an Ambion Northern Max-Gly Kit (Austin, TX, USA). Total RNA (30 μg) was electrophoresed on a 1% agarose gel containing 0.4 M formaldehyde, transferred to a nylon membrane, and then fixed by UV crosslinking. Then, the membrane was prehybridized with UltraHybOligo Hybridization Bufer (Thermo Fisher Scientific) and hybridized with precursor miR-92b-specifc oligonucleotide probes labeled with digoxigenin-ddUTP in roller bottles. The quantitative data were detected by Gel-Pro Analyzer 4.0 software (Media Cybernetics, MD).

Statistical analysis

Two groups (unpaired t-test), multiple groups (analysis of variance (ANOVA) Tukey's test) and paired tissues (paired t-test) were compared. Differences between paired tissues were compared using paired t-test. The 62 GC patients were divided into two circ-MYBL2 and precursor miR-92b groups (low and high, n=31). Associations between patients' clinical data and levels of circ-MYBL2 and precursor miR-92b were analyzed with Chi squared test. A $P<0.05$ was considered statistically significant.

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Results

MiR-92b and circ-MYBL2 expression in GC and their correlation

Our preliminary deep sequencing determined the accumulation of circ-MYBL2 in 12 pairs of cancer and non-cancer tissue samples from 12 GC patients. Accumulation of circ-MYBL2 was normalized to reads per million (rpm). As shown in Supplemental Table 1, rpm of circ-MYBL2 was lower in cancer tissues than in non-cancer tissues in all 12 cases. Therefore, circ-MYBL2 is likely involved in GC. Expression of miR-92b and circ-MYBL2 in paired tissues from 62 GC patients included in the present research was further quantified using RT-qPCR. The results showed that mature miR-92b expression was significantly increased (Fig. 1A, $p<0.01$) while precursor miR-92b (Fig. 1B, $p<0.01$) and circ-MYBL2 expression were downregulated (Fig. 1C, $p<0.01$) in GC tissues. Pearson's correlation analysis revealed that circ-MYBL2 level was inversely correlated with mature miR-92b (Fig. 1D) but positively correlated with precursor miR-92b (Fig. 1E). Moreover, RT-qPCR were also performed to analyze the expression of both mature and precursor miR-92b and circ-MYBL2. Mature miR-92b was upregulated while precursor miR-92b and circ-MYBL2 levels were downregulated in GC cell lines (Fig. 1F, $p<0.01$). Chi squared test showed that precursor miR-92b and CircRNA circ-MYBL2 were only closely associated with tumor size, but not other clinical factors (Table 1).

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Subcellular location of circ-MYBL2 and its potential interaction with miR-92b

Subcellular location of circ-MYBL2 in nuclear (N) and cytoplasmic (C) fractions

in both MKN-28 and BGC-823 cells was analyzed. Similar to GAPDH, it was observed that circ-MYBL2 was detected in both N and C fractions (Fig. 2A). It is worth noting that the same value of parameters (Tm, product length, GC content) were used to design primers for both GAPDH and circ-MYBL2. So, the PCR products of circ-MYBL2 and GAPDH were with similar length. Please check Supplemental Fig. 1 for gel images of all three replicates. IntaRNA 2.0 prediction revealed multiple potential base pairs formed by circ-MYBL2 (Fig. 2B). RNA pulldown assay showed that circ-MYBL2 level was significantly higher in Bio-miR-92b (pre) group, suggesting the binding of miR-92b to circ-MYBL2 (Fig. 2C, $p<0.001$).

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Role of circ-MYBL2 in miR-92b maturation in MKN-28 and BGC-823 cells

Circ-MYBL2 or miR-92b were transfected in MKN-28 and BGC-823 cells followed by RT-qPCR every 24h until 72h to confirm the overexpression of circ-MYBL2 and miR-92b (Fig. 3A, $p<0.05$). Interestingly, circ-MYBL2 overexpression increased precursor miR-92b expression (Fig. 3B, $p<0.05$) but inhibited miR-92b in both MKN-28 and BGC-823 cells (Fig. 3C, $p<0.05$). Northern blotting results revealed that precursor miR-92b was significantly upregulated in MKN-28 and BGC-823 cells transfected circMYBL2 (Fig. S1). Moreover, RT-qPCR assay was used to detect the expression of miR-92b maturation in MKN-28 and BGC-823 cells transfected with si-circMYBL2. The results revealed that circMYBL2 knockdown enhanced the expression of miR-92b maturation (Fig. S2). It is worth noting that no potential

binding site of circ-MYBL2 on the promoter region of miR-92b gene was predicted. Therefore, the increased accumulation of precursor miR-92b was likely caused by the reduced maturation.

Effects of circ-MYBL2 and miR-92b on the proliferation of MKN-28 and BGC-823 cells

BrdU assay showed that circ-MYBL2 overexpression decreased cell proliferation while increased miR-92b (mature) promoted cell proliferation. Moreover, circ-MYBL2 overexpression suppressed the effect of miR-92b on cell proliferation (Fig. 4A, $p<0.05$). The effect of circ-MYBL2 and miR-92b on the growth of GC tumor xenograft of MKN-28 cells was analyzed using *in vivo* tumor xenograft assay. Tumors were collected at 4 weeks after injection (Fig. 4B). Tumor volumes were calculated and compared. Circ-MYBL2 suppressed tumor growth while miR-92b promoted tumor growth. Moreover, circ-MYBL2 suppressed the effect of miR-92b on tumor growth (Fig. 4C, $p<0.05$). It is worth noting that the role of circ-MYBL2 in many behaviors of GC cells such as invasion, migration, apoptosis and proliferation was explored. It seems that circ-MYBL2 only affect the proliferation of GC cells.

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Discussion

The crosstalk between circ-MYBL2 and miR-92b was explored in this study. Our study first illustrated the altered expression of circ-MYBL2 and miR-92b in GC. We also showed that circ-MYBL2 could act as a molecular sponge of precursor miR-92b to inhibit the production of mature miR-92b.

Recent studies reported the involvement of circ-MYBL2 in both multiple myeloma and cervical cancer.[19, 20] However, the function of circ-MYBL2 in these two cancers are opposite. In multiple myeloma, circ-MYBL2 is downregulated and predicts poor survival. In addition, circ-MYBL2 overexpression increases the binding of MYBL2 with cyclin F, thereby downregulating a considerable number of oncogenes involved in cell proliferation. These results suggest the tumor-suppressive role of circ-MYBL2 in multiple myeloma.[19] In contrast, circ-MYBL2 is highly expressed in cervical cancer and it sponges miR-361-3p to increase cell invasion and proliferation, indicating its oncogenic role.[20] Our study showed that circ-MYBL2 was downregulated in GC and its overexpression significantly suppressed the proliferation of two GC cell lines. We concluded that circ-MYBL2 is likely a tumor-suppressive circRNA in GC. Therefore, functional characterization of circ-MYBL2 in other cancer is recommended.

MiR-92b also plays different roles in different cancers. In gastric cancer, miR-62b is overexpressed, and it can activate PI3K/AKT signaling pathway via

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DAB2IP to promote cancer cell proliferation.[21] In contrast, miR-92b is under-expressed in breast cancer, and it targets EZH2 to increase autophagy and reduce cell invasion and viability.[22] Consistently, our study also reported the upregulation of miR-92b in GC and its enhancing effects on cell proliferation. The upstream regulator of miR-92b in cancer biology remains largely unknown. Our study showed that precursor miR-92b could bind to circ-MYBL2, and circ-MYBL2 overexpression upregulated precursor miR-92b level and downregulated mature miR-92b level. Moreover, circ-MYBL2 could be detected in both nucleus and cytoplasm. Thus, circ-MYBL2 in the nucleus (precursor miRNAs are in nucleus) may sponge precursor miR-92b to inhibit its movement from the nucleus to the cytoplasm, thereby suppressing its maturation. This is because the movement of precursor miRNAs out of nucleus is required for their maturation. It is known that it circ-MYBL2 can sponge miR-361-3p in cytoplasm of cervical cancer cells.[16] Therefore, circ-MYBL2 may serve as an upstream inhibitor of miR-92b by suppressing its maturation. Our study showed that circ-MYBL2 can sponge precursor miR-92b in nucleus. Therefore, circ-MYBL2 in different subcellular locations may have different functions.

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To date, the role of circRNAs in the maturation of miRNAs have not been well studied. Our study is the first to report that the maturation of miR-96b can be regulated by a circRNA. Other circRNAs may have similar roles in the maturation of miRNAs. More studies are needed to explore the role of circRNAs in this process.

However, the present research is limited by the small sample size, and the conclusions should be verified by studies with more patients. In addition, the diagnostic and prognostic values of miR-92b and Circ-MYBL2 for GA should also be studied.

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Conclusions

Circ-MYBL2 was under-expressed in GC, and it might sponge precursor miR-92b to suppress its maturation, thereby inhibiting GC cell proliferation.

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Authors' contributions

Ruijie Luo: study concepts, literature research, clinical studies, data analysis, experimental studies, manuscript writing and review.

Conflicts of interest

The authors have no conflict of interest.

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Not Applicable.

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Figure legends

Figure 1 Quantification of miR-92b and circ-MYBL2 expression in GC and analysis of their correlations. The expression of mature (A) and precursor (B) miR-92b and circ-MYBL2 (C) was quantified using RT-qPCR. The correlations between circ-MYBL2 and mature miR-92b (D) or precursor miR-92b (E) were analyzed using Pearson's correlation coefficient. The expression of miR-92b (both mature and precursor) and circ-MYBL2 in two GC cell lines MKN-28 and BGC-823 and normal gastric epithelial cell line GES-1 (F) was also analyzed using RT-qPCR. **, $p<0.01$. Abbreviations: GC, gastric cancer; RT-qPCR, reverse transcription polymerase chain reaction.

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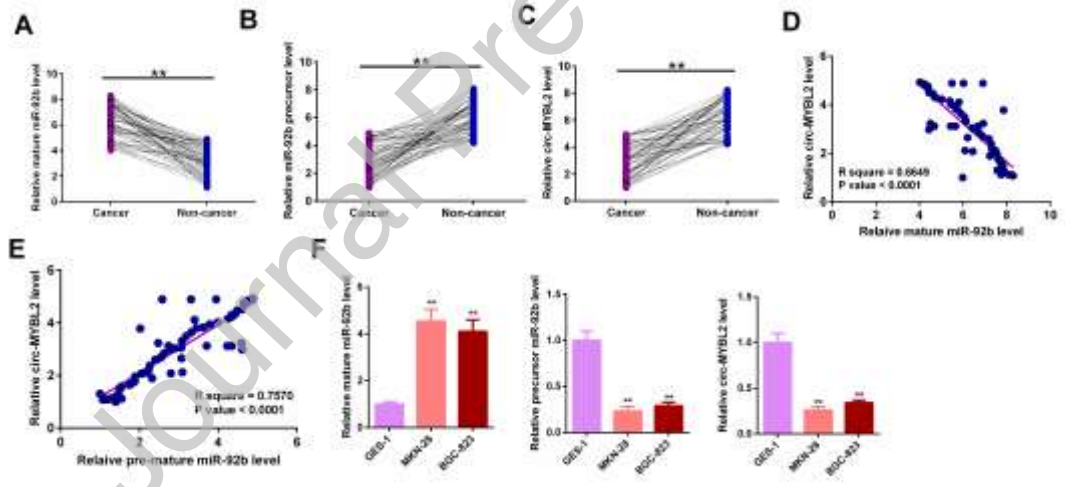

Figure 2 The binding of miR-92b to circ-MYBL2. Subcellular location of circ-MYBL2 (A) in MKN-28 and BGC-823 cells was analyzed using nuclear (N) and cytoplasmic (C) fraction assay. The potential base pairing between circ-MYBL2 and miR-92b (B) was predicted using IntaRNA 2.0. The direct interaction between precursor miR-92b and circ-MYBL2 (C) was confirmed using RNA pulldown assay. ***, $p<0.001$. Abbreviations: N, nucleus; C, cytoplasm; Pre, precursor.

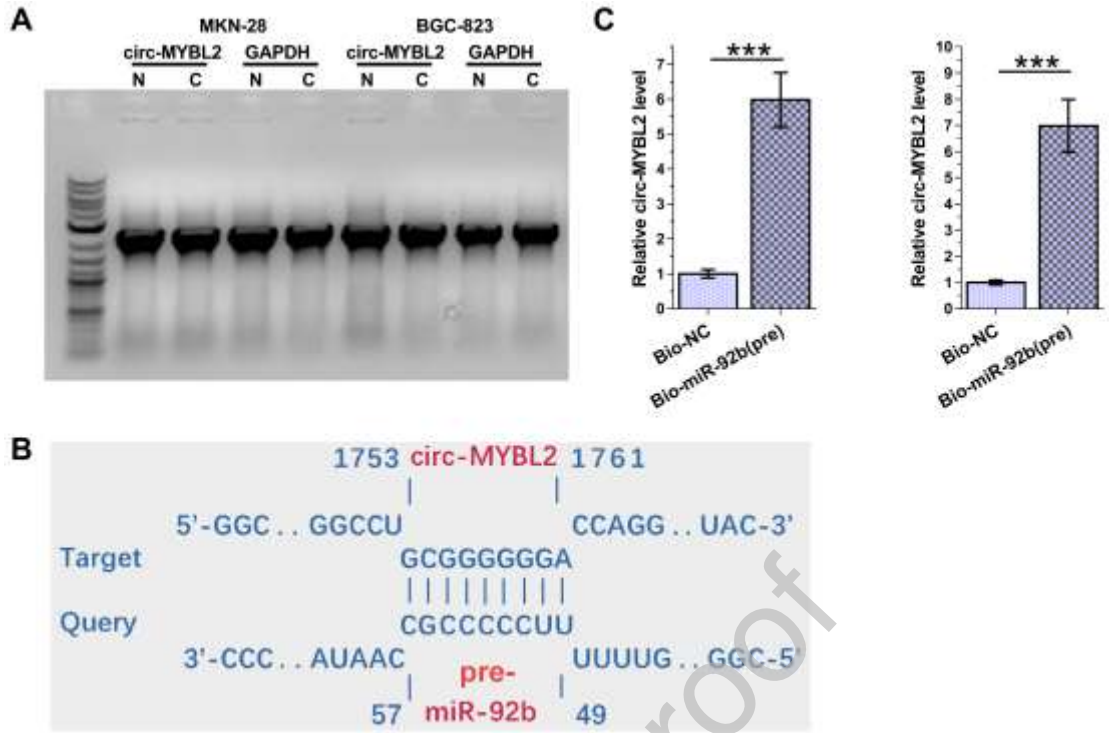

Figure 3 Role of circ-MYBL2 in miR-92b maturation.

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Circ-MYBL2 or miR-92b were overexpressed in MKN-28 and BGC-823 cells, followed by the confirmation of circ-MYBL2 and miR-92b overexpression using RT-qPCR every 24h until 72h (A). The regulatory effects of circ-MYBL2 on miR-92b expression at precursor (B) and mature levels (C) were analyzed using RT-qPCR. *, $p<0.01$. Abbreviations: RT-qPCR, reverse transcription polymerase chain reaction.

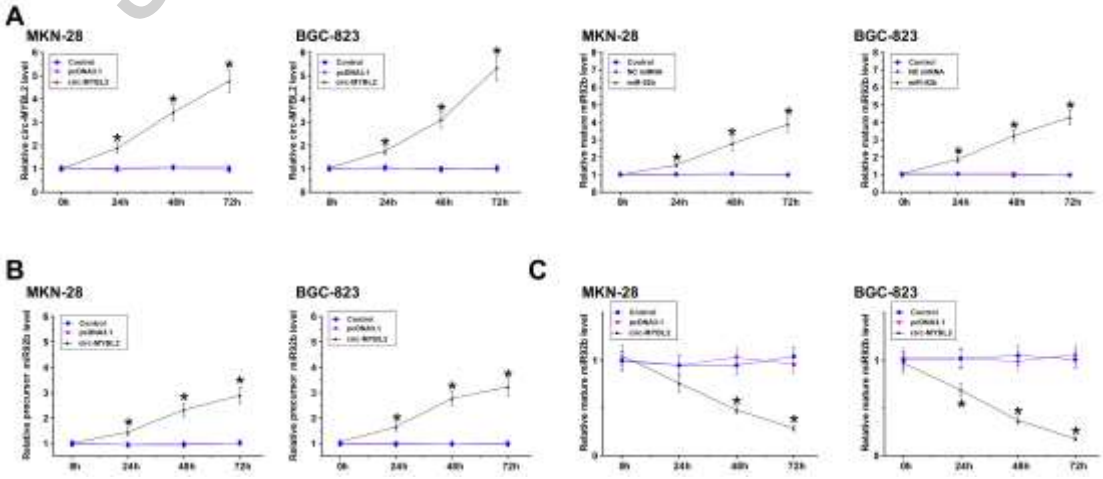

Figure 4 Roles of circ-MYBL2 and miR-92b in the proliferation of MKN-28 and BGC-823 cells. MKN-28 and BGC-823 cells were subjected to BrdU assay to analyze the role of circ-MYBL2 and miR-92b in the proliferation of MKN-28 and BGC-823 cells (A). *In vivo* tumor xenograft assay was performed using MKN-28 cells to explore the role of circ-MYBL2 and miR-92b in *in vivo* growth of GC tumors (B). Tumor volumes were calculated and compared (C). Tumor are form two different figures. These two figures were merged by normalizing tumor size to the same ruler scale. *, $p<0.05$. Abbreviations: GC, gastric cancer.

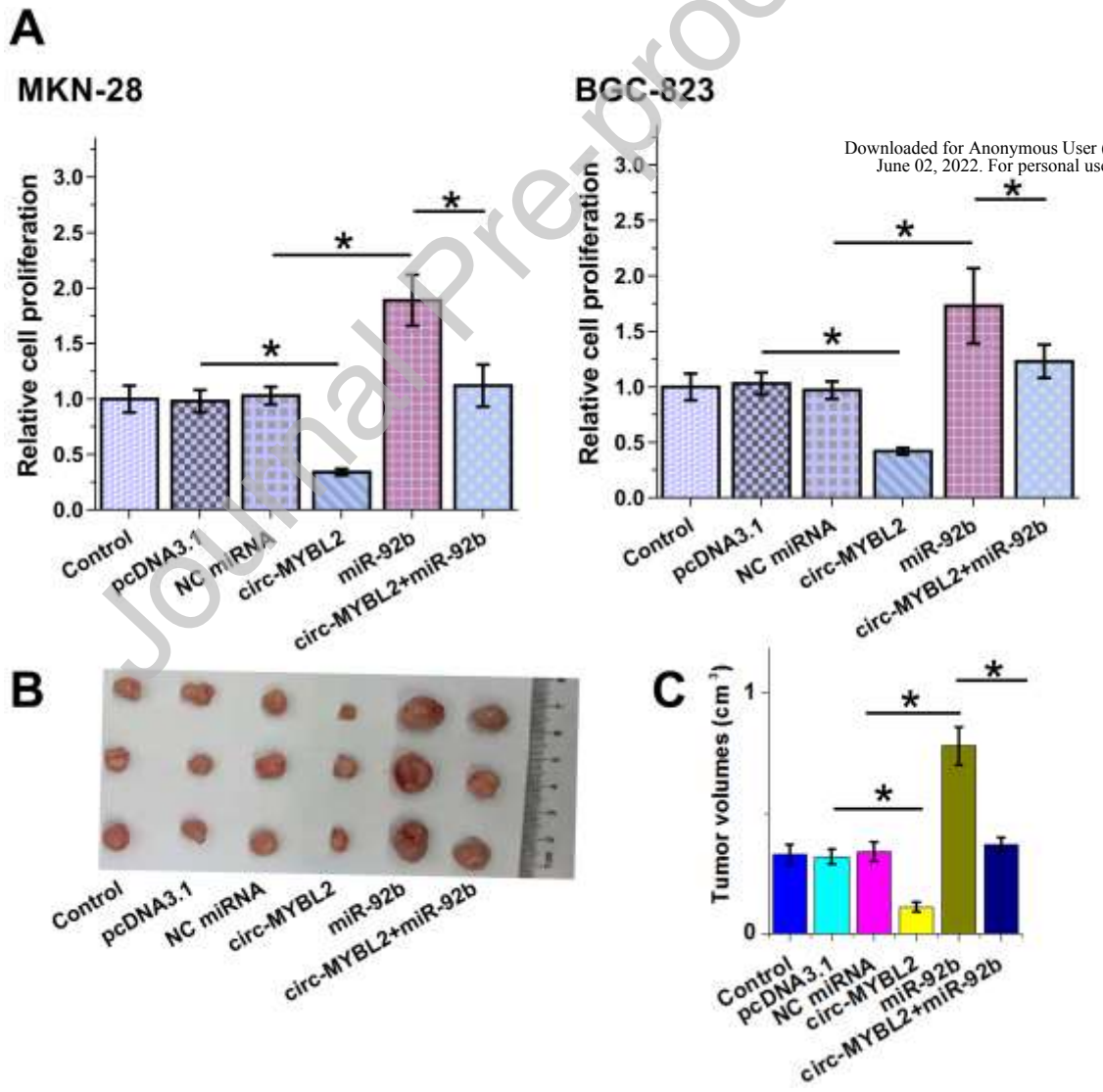

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Table 1 Clinicopathological data of 62 patients.

Index		Number	%
Sex			
	Male	38	61.29%
	Female	24	38.71%
Age			
	<55	20	32.26%
	⩾55	42	67.74%
Primary tumor			
	T1-2	15	24.19%
	T3-4	47	75.81%
Borrmann classification			
	I-II	48	77.42%
	III-IV	14	22.58%
Lymph nodes			
	N0-1	19	30.65%
	N2-3	43	69.35%
Metastasis			
	M0	52	83.87%
	M1	10	16.13%
Clinical stage			
	I-II	28	45.16%
	III-IV	34	54.84%

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