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CircRNA ZFR stimulates the proliferation of hepatocellular carcinoma through upregulating MAP2K1

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Abstract. – OBJECTIVE: The aim of the study was to illustrate the function of circRNA ZFR in aggravating the development of hepatocellular carcinoma (HCC) by upregulating MAP2K1.

PATIENTS AND METHODS: CircRNA ZFR levels in 62 paired HCC and paracancerous species were detected. The influence of circRNA ZFR on clinical data of HCC patients was analyzed. After the overexpression or knockdown of circRNA ZFR, changes in viability and clonality of Bel-7402 and Hep3B cells were assessed, respectively. The involvement of circRNA ZFR/MAP2K1 axis in the development of HCC was explored through Luciferase assay and rescue experiments.

RESULTS: CircRNA ZFR was highly expressed in HCC species than the paracancerous ones. Higher level of circRNA ZFR predicted more advanced tumor grading of HCC. The knockdown of circRNA ZFR attenuated the proliferative ability of HCC cells, while the overexpression of circRNA ZFR obtained opposite results. MAP2K1 level was positively correlated to that of circRNA ZFR. Luciferase assay uncovered that circRNA ZFR can be targeted by MAP2K1 through specific binding sites. In addition, the overexpression of MAP2K1 could reverse the influence of silenced circRNA ZFR on proliferative ability of HCC cells.

CONCLUSIONS: CircRNA ZFR is upregulated in HCC and closely linked to tumor grading. It promotes proliferative ability in HCC by upregulating MAP2K1.

Key Words:
CircRNA ZFR, MAP2K1, HCC, Proliferation.

Introduction

Hepatocellular carcinoma (HCC) is one of the most popular malignancies in the world. Similar to other malignancies, inactivation of tumor-suppressor genes and/or the multi-step, multi-stage process of proto-oncogene activation are responsible for the etiology of HCC[1-3]. It is currently believed that viral hepatitis, alcohol abuse, and non-alcoholic hepatic steatosis are the main causes of HCC[3,4]. The incidence of HCC is concealed, and most patients are already in the advanced stage at the first time of diagnosis. The commonly used imaging examination and serum alpha-fetoprotein (AFP) detection have limited diagnostic abilities for early-stage HCC. The overall prognosis of HCC is extremely poor, and the 5-year survival rate is low[4-6]. Therefore, actively seeking effective diagnosis and treatment is of significance[1,3]. Recently, circRNAs have been highlighted in the field of molecular biology. They exert a good application prospect in the diagnosis, treatment, and prognosis of tumor diseases[7,8].

CircRNAs are a class of endogenous non-coding RNAs that differ from linear RNAs in a closed loop structure[9,10]. Compared to linear RNA, it has no 5' end cap and 3' end poly A tail[11,12]. CircRNAs are widely expressed in eukaryotic cells and conserved among different species. They are not susceptible to RNase degradation, which are stable biomarkers applied in disease diagnosis[12-14]. With the rapid development of second-generation sequencing and bioinformatics technology, thousands of circRNAs have been discovered. The abundances of some circRNAs are more than 10 times of their corresponding linear RNAs[12,15,16]. It is reported that circRNAs may exert transcriptional and post-transcriptional regulation in organ development and disease progression[16]. CircRNA ZFR has been previously reported [17,18]. Bioinformatics analysis uncovered that circRNA ZFR specifically bound to MAP2K1. Currently, the vital function of MAP2K1 in the development of a variety of human tumors has been identified[19,20]. This experiment comprehensively analyzed the expressions and biological effects of circRNA ZFR and MAP2K1 in the development of HCC.

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Patients and Methods

HCC Species

A total of 62 paired invasive HCC and paracancerous species were collected and stored at -80°C. None of the enrolled subjects were preoperatively treated with anti-tumor therapy. Their clinical data were recorded. Patients and their families in this study have been fully informed. In this study, tumor staging was assessed based on the guideline proposed by the Union for International Cancer Control (UICC). This study was approved by Ethics Committee of Zhongnan Hospital of Wuhan University. This study was conducted in accordance with the Declaration of Helsinki.

Cell Culture

Six HCC cell lines (Bel-7402, HepG2, MHCC88H, SMMC-7221, Huh7, Hep3B) and one normal hepatocyte cell line (LO2) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cell passage was conducted in 1×typsin+EDTA at 80-90% confluence.

Transfection

Until cells were grown to 30-50% of cell density, they were transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfected cells for 48 h were harvested for functional experiments.

Cell Counting Kit-8 (CCK-8) Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the CCK-8 kit (Dojindo Molecular Technologies, Kumamoto, Japan) for plotting the viability curves.

Colony Formation Assay

Cells were inoculated in a 6-well plate with 200 cells per well and cultured for 2 weeks. Culture medium was replaced once in the first week and twice in the second week. Visible colonies were washed in phosphate buffered saline (PBS), fixed in methanol for 20 min, and dyed in 0.1% crystal violet for 20 min, which were captured and calculated.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. CircRNA ZFR: forward: 5'-AACCACCACAGATTCACTAT-3', reverse: 5'-AACCACCACAGATTCACTAT-3'; MAP2K1: forward: 5'-CAAGAAGAAGCCGACGCCCAT-3', reverse: 5'-GACGCCAGCAGCATGGGTTG-3'; GAPDH: forward: 5'-ATAGCACAGCCTGGATAGCAACGTAC-3', reverse: 5'-CACCTTCTACAATGAGCTGCGTGTG-3'.

Luciferase Assay

Cells inoculated in a 24-well plate were co-transfected with circRNA ZFR-WT/circRNA ZFR-MUT and NC/pcDNA-MAP2K1, respectively. 48 hours later, the cells were lysed for determining the relative Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between two groups were analyzed by the *t*-test. Correlation between expressions of two genes was compared by Pearson correlation test. $p<0.05$ was considered as statistically significant.

Results

CircRNA ZFR Was Highly Expressed in HCC

Differential expressions of circRNA ZFR in HCC and paracancerous species were detected by qRT-PCR. It is shown that circRNA ZFR was highly expressed in HCC species (Figure 1A). In the same way, it was upregulated in HCC cell lines compared to that of LO2 cells (Figure 1B).

CircRNA ZFR Was Correlated to Tumor Staging of HCC

Enrolled HCC patients were assigned into two groups based on the cut-off value of circRNA

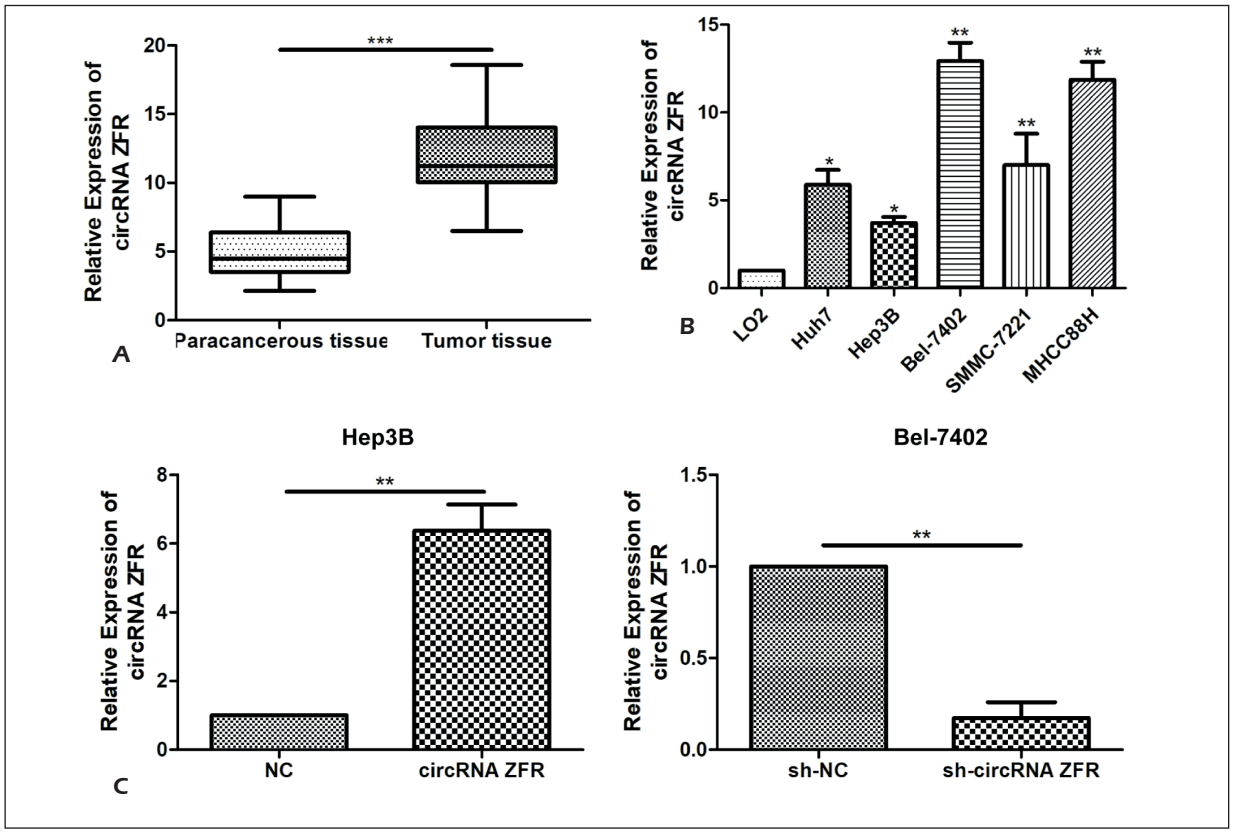

Figure 1. CircRNA ZFR was highly expressed in HCC. **A**, Differential expressions of circRNA ZFR in HCC species and paracancerous ones. **B**, CircRNA ZFR levels in HCC cell lines. **C**, Transfection efficacy of sh-circRNA ZFR and pcDNA-circRNA ZFR in Bel-7402 and Hep3B cells, respectively. Data were expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

ZFR. By analyzing their clinical data, it is found that circRNA ZFR level was positively correlated to tumor staging, while it was not related to age, sex, rates of distant metastasis and lymphatic metastasis of HCC patients (Table I).

CircRNA ZFR Promoted Proliferative Ability in HCC

To explore the biological function of circRNA ZFR, overexpression and knockdown models of circRNA ZFR were constructed in Hep3B and Bel-7402 cells, respectively. Transfection efficacy of both models was excellent (Figure 1C). Overexpression of circRNA ZFR enhanced viability and colony number in Hep3B cells, while the knockdown of circRNA ZFR in Bel-7402 cells obtained the opposite trends (Figure 2A, 2B).

MAP2K1 Was Lowly Expressed in HCC

Through bioinformatics analysis, five potential miRNAs binding circRNA ZFR were predicted. By detecting their levels in overexpression and knockdown models of circRNA ZFR, MAP2K1 expression showed the most pronounced change (Figure 3A). MAP2K1 was upregulated in HCC species (Figure 3B) and cell lines (Figure 3F). Besides, its level was positively correlated to that of circRNA ZFR in HCC species (Figure 3C). Overexpression of circRNA ZFR in Hep3B cells upregulated MAP2K1 level, and the knockdown of circRNA ZFR downregulated its level in Bel-7402 cells (Figure 3D). Similarly, MAP2K1 could positively regulate circRNA ZFR level in HCC cells as well (Figure 3E). Decreased Luciferase activity was identified after co-transfection of circRNA ZFR-WT and pcDNA-MAP2K1, verifying the binding between circRNA ZFR and MAP2K1 (Figure 3G).

CircRNA ZFR Modulated the Development of HCC by Upregulating MAP2K1

Downregulated circRNA ZFR in Hep3B cells transfected with sh-circRNA ZFR was upregulated by overexpression of MAP2K1. Meanwhile, the knockdown of MAP2K1 reduced the upregulated circRNA ZFR in Bel-7402 cells overexpressing circRNA ZFR (Figure 4A).

Table I. Association of circRNA ZFR and MAP2K1 expression with clinicopathologic characteristics of hepatocellular carcinoma.

Parameters	Number of cases	CircRNA ZFR expression		*p*-value	MAP2K1 expression		*p*-value
		Low (%)	High (%)		Low (%)	High (%)	
Age (years)				0.123			0.638
<60	26	16	10		16	10	
≥60	36	15	21		20	16	
Gender				0.446			0.607
Male	31	17	14		19	12	
Female	31	14	17		17	14	
T stage				0.010			0.033
T1-T2	36	23	13		25	11	
T3-T4	26	8	18		11	15	
Lymph node metastasis				0.189			0.210
No	39	22	17		25	14	
Yes	23	9	14		11	12	
Distance metastasis				0.123			0.274
No	36	21	15		23	13	
Yes	26	10	16		13	13	

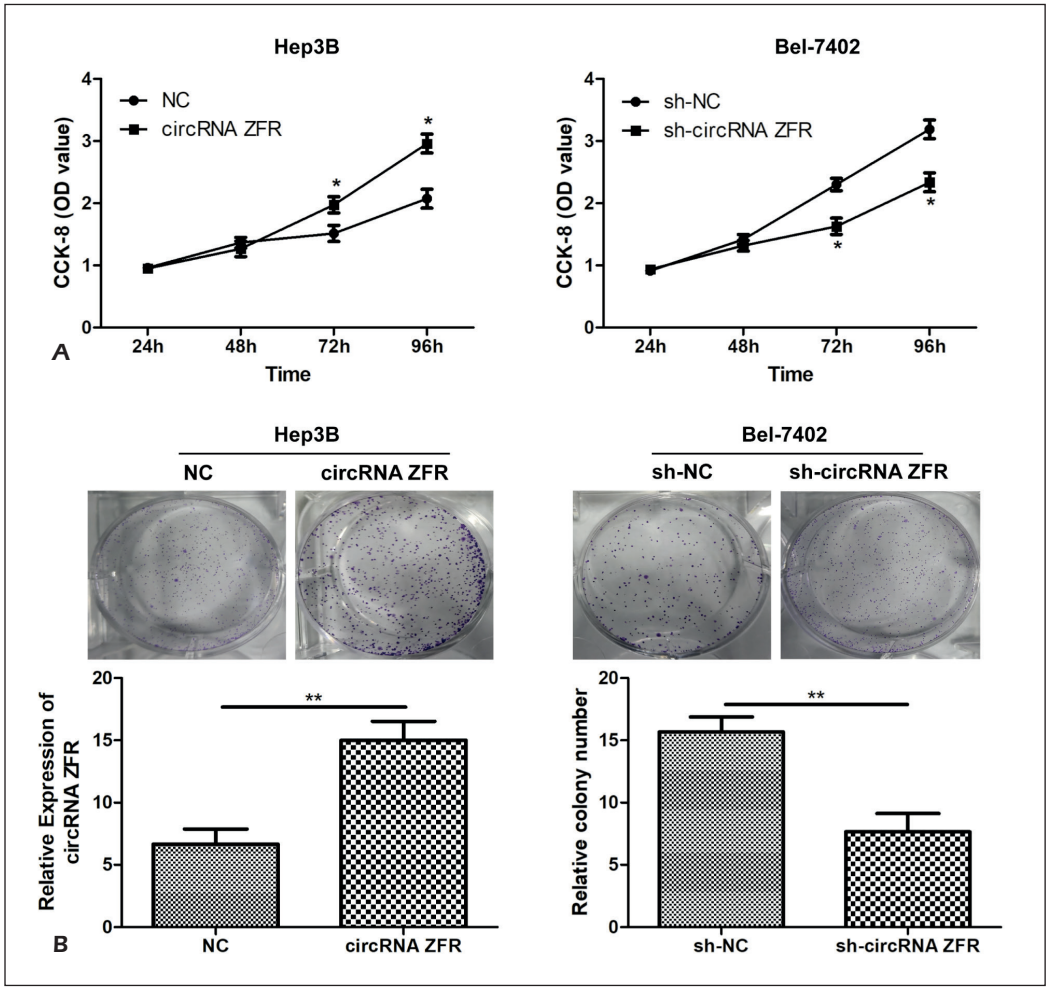

Figure 2. CircRNA ZFR promoted proliferative ability in HCC. **A**, CCK-8 assay showed viability in Hep3B and Bel-7402 cells with overexpression or knockdown of circRNA ZFR. **B**, Colony formation assay showed colony number in Hep3B and Bel-7402 cells with overexpression or knockdown of circRNA ZFR (magnification: 10 ×). Data were expressed as mean±SD. **p*<0.05, ***p*<0.01.

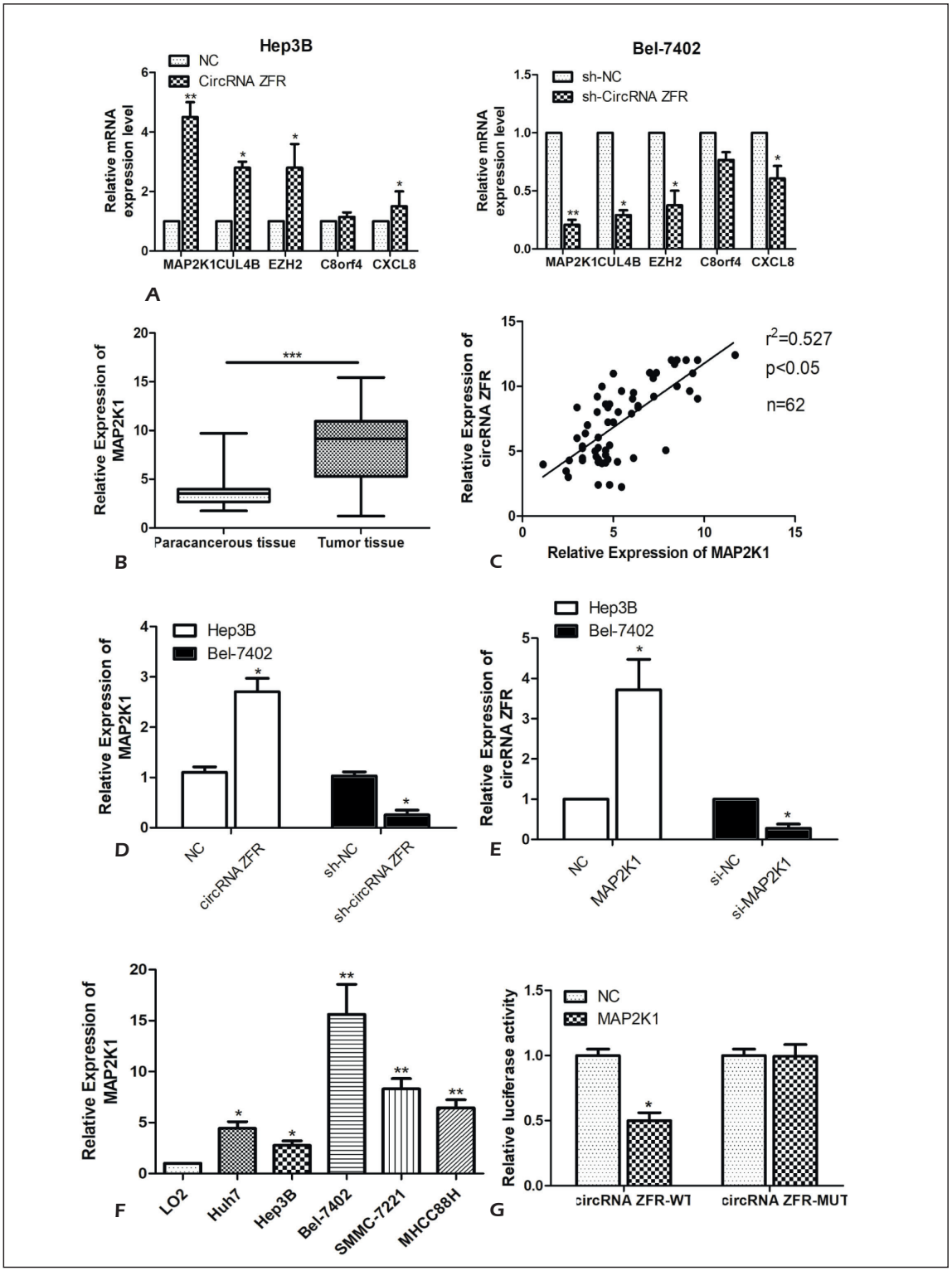

Figure 3. MAP2K1 was lowly expressed in HCC. **A**, Expression levels of five potential miRNAs binding circRNA ZFR in Hep3B and Bel-7402 cells with overexpression or knockdown of circRNA ZFR. **B**, Differential expressions of MAP2K1 in HCC species and paracancerous ones. **C**, A positive correlation between expressions of circRNA ZFR and MAP2K1 in HCC species. **D**, MAP2K1 level in Hep3B and Bel-7402 cells with overexpression or knockdown of circRNA ZFR. **E**, CircRNA ZFR level in Hep3B and Bel-7402 cells with overexpression or knockdown of MAP2K1. F, MAP2K1 levels in HCC cell lines. **G**, Luciferase activity after co-transfection with circRNA ZFR-WT/ circRNA ZFR-MUT and NC/pcDNA-MAP2K1, respectively. Data were expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

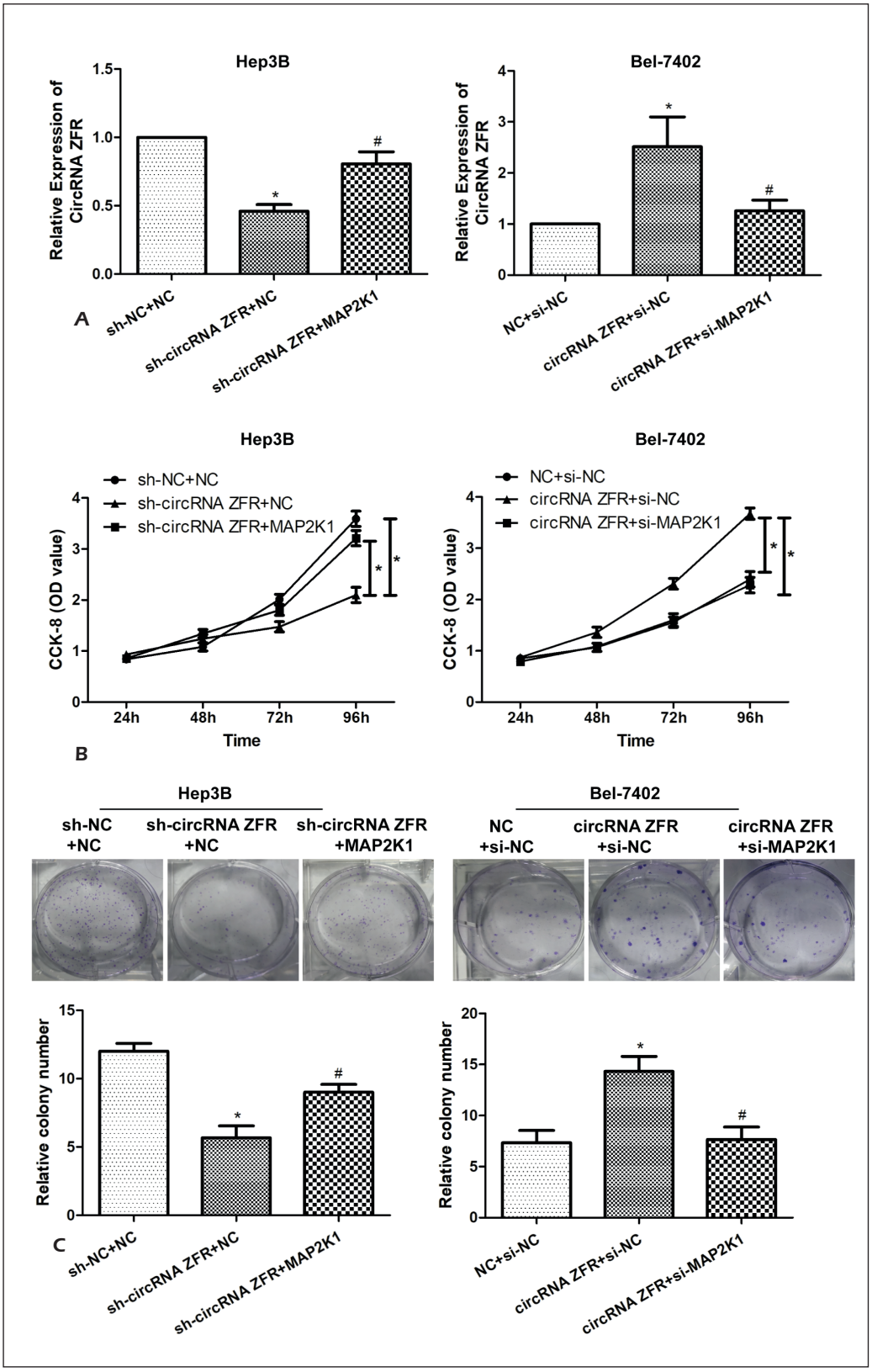

Figure 4. CircRNA ZFR modulated the development of HCC by upregulating MAP2K1. **A**, CircRNA ZFR level in co-transfected Hep3B and Bel-7402 cells. **B**, Viability in co-transfected Hep3B and Bel-7402 cells. **C**, Colony number in co-transfected Hep3B and Bel-7402 cells (magnification: 10 ×). Data were expressed as mean±SD. *p<0.05.

Notably, inhibitory effect of silenced circRNA ZFR on Hep3B cell proliferation was reversed by overexpression of MAP2K1. Stimulated viability and clonality owing to overexpression of circRNA ZFR were abolished by knockdown of MAP2K1 (Figure 4B, 4C).

Discussion

HCC is featured by insidious onset and high degree of malignancy. Timely diagnosis and early treatment can significantly prolong the survival of HCC[3-5]. Diagnosis and screening of HCC are mainly based on imaging examinations and detection of tumor biomarkers. The latter greatly contributes to improve therapeutic efficacy and monitor the recurrence of HCC[6,7]. At present, tumor biomarkers have some limitations, such as high false positive rate, and low sensitivity and specificity[7,8]. Development of HCC-related biomarkers is urgently required.

CircRNA is a type of single-stranded closed circular non-coding RNA that has neither the 5' end nor the 3' end poly A tail[9-11]. Due to its special stable structure, circRNA is highly conserved in evolution[12-14]. CircRNA ZFR is a newly discovered one, and it is differentially expressed in numerous tumors[17,18]. Therefore, the objective of this study was firstly to elucidate the oncogenic role of circRNA ZFR in the progression of HCC, as well as the specific mechanism of circRNA ZFR regulating MAP2K1. In this study, our findings revealed that circRNA ZFR was upregulated in HCC tissues and cell lines. Besides, higher level of circRNA ZFR predicted more advanced tumor grading of HCC patients. *In vitro* experiments verified that the knockdown of circRNA ZFR attenuated proliferative ability of HCC cells, while the overexpression of circRNA ZFR obtained the opposite result.

CircRNAs exert their critical functions by sponging miRNAs as ceRNAs[21,22]. Previous investigations predicted binding sequences in the 3'UTR of circRNA ZFR and MAP2K1, and our Luciferase assay further detected this finding. MAP2K1 level was positively correlated to that of circRNA ZFR in HCC species. Luciferase assay uncovered that circRNA ZFR can be targeted by MAP2K1 through specific binding sites. In addition, the overexpression of MAP2K1 could reverse the influence of silenced circRNA ZFR on proliferative ability of HCC cells. As a result, we believed that circRNA ZFR stimulated the malignant progression of HCC by upregulating MAP2K1.

Conclusions

Briefly, circRNA ZFR is upregulated in HCC and closely linked to tumor grading. It promotes proliferative ability in HCC by upregulating MAP2K1.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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