Heliyon 10 (2024) e28785

Contents lists available at ScienceDirect

Heliyon

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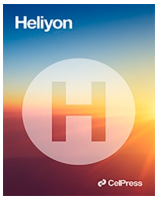

Research article

CircRNA circRREB1 promotes tumorigenesis and progression of breast cancer by activating Erk1/2 signaling through interacting with GNB4

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A R T I C L E I N F O

Keywords:
CircRREB1
RNA-Binding protein
GNB4
Erk1/2 signaling
Breast cancer

A B S T R A C T

Current investigations have illuminated the essential roles played by circular RNAs (circRNAs) in driving breast cancer (BC) tumorigenesis. However, the functional implications and molecular underpinnings of most circRNAs in BC are not well characterized. Here, Circular RNA (circRNA) expression profiles were analyzed in four surgically resected BC cases along with adjacent non-cancerous tissues applying RNA microarray analysis. The levels and prognostic implications of circRREB1 in BC were subjected to quantitative real-time PCR (qRT-PCR) and in situ hybridization (ISH). Experimental manipulation of circRREB1 levels in both *in vivo* and *in vitro* settings further delineated its role in BC cell growth, invasion, and metastasis. The mechanical verification of circRREB1's interaction with GNB4 was established through RNA pull-down, mass spectrometry, Western blot analysis, RNA immunoprecipitation assays (RIP), fluorescence ISH (FISH), and rescue experiments. We found that circRREB1 exhibited significant upregulation in BC tissues and cells, implicating its association with an unfavorable prognosis in BC patients. CircRREB1 knockdown elicited anti-proliferative, anti-migratory, anti-invasive, and pro-apoptotic effects in BC cells, whereas its upregulation exerted opposing influences. Follow-up mechanistic examinations suggested that circRREB1 might interact with GNB4 directly, inducing the activation of Erk1/2 signaling and driving BC progression. Our findings collectively indicate that the interplay of circRREB1 with GNB4 promotes Erk1/2 signaling, thereby fostering BC progression, and positioning circRREB1 as a candidate therapeutic target for intervention in BC.

1. Introduction

Among women, breast cancer (BC) holds the highest prevalence among malignancies, representing 31% of all tumors, and exhibits an inclination towards younger age groups based on 2022 cancer data [1]. Studies have shown that roughly 1.67 million women receive a BC diagnosis every year, with approximately 500,000 succumbing to the disease [2]. Also, with the global trend towards longer life expectancy, there is a projected rise in both the prevalence and mortality rates of BC [3]. Despite new therapeutic avenues,

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https://doi.org/10.1016/j.heliyon.2024.e28785
Received 15 June 2023; Received in revised form 24 March 2024; Accepted 25 March 2024
Available online 4 April 2024

tumor metastasis and recurrence remain challenging in BC treatment [4]. Therefore, the search for molecular biomarkers tightly correlated with BC initiation and advancement is pressing, aiming to revolutionize both diagnosis and treatment avenues.

CircRNAs differ from linear RNA molecules in their closed-loop structure, which arises from the back-splicing of precursor messenger RNAs without 5′ caps and 3′ tails [5]. CircRNAs, pivotal regulators, are intricately involved in the development and progression of a range of human diseases, including cancer, eliciting substantial attention [6]. CircRNAs perform their biological functions mainly by acting as miRNA sponges, transcriptional regulators, templates for protein translation, sequestering agents for RNA-binding protein, etc. [7]. However, underlying biological functions of interactions between circRNAs and proteins are rarely reported.

Emerging research has highlighted the involvement of aberrantly expressed circRNAs in the development and advancement of several cancer types. It is widely accepted that circRNAs predominantly serve as miRNA decoys, counteracting the repressive effects on gene expression exerted by miRNAs [8–10]. For example, circRNF20 boosts BC progression and the Warburg effect via miR-487-a/HIF-1α/HK2 [11]. Hsa_circ_001783 exhibits significant upregulation and correlates with unfavorable prognosis in BC patients, exerting its oncogenic role by sequestering miR-200c-3p [12]. Another research has shown that by sequestering miR-208a and miR-3164, circRAD18 fosters BC advancement through the elevation of IGF1 and FGF2 expression [13]. Emerging research suggests that circRNA binding proteins (RBPs) are central players in the regulation of circRNA synthesis and degradation, with implications for protein expression, biogenesis, and pathophysiological pathways arising from circRNA-protein interactions [14,15]. In a specific case, circ0005276, by interaction with FUS, is implicated in enhancing the growth and metastasis of prostate cancer cells and subsequently activating XIAP transcription [16]. By binding to HuR, CircRHOBTB3 promotes β-Trcp1-mediated ubiquitination, thereby suppressing colorectal cancer (CRC) aggressiveness [17]. CircURI1 interacts with hnRNPM, influencing gene alternative splicing and cell migration, consequently inhibiting gastric cancer metastasis [18]. The involvement of circMYBL2 in BC initiation and aggressiveness was elucidated through its interaction with the circMYBL2/miR-1205/E2F1 and circMYBL2/eIF4A3/E2F1 signaling cascades [19]. A novel feedback loop, FUS/circEZH2/KLF5/CXCR4, was identified by Liu et al. [20], who propose circEZH2 as a promising biomarker and therapeutic target for BC patients. A novel feedback loop, FUS/circEZH2/KLF5/CXCR4, was identified by Liu et al. [20] who propose circEZH2 as a promising biomarker and therapeutic target for BC patients. Furthermore, a recent literature review has explored the origin, roles, and clinical relevance of circular RNAs in BC, emphasizing their potential as targets for diagnosis and therapy [21]. However, the specific interactions between circRNAs and proteins, along with their underlying molecular mechanisms, remain poorly understood in BC.

G protein-mediated activation of diverse cellular signal cascades by G protein-coupled receptors (GPCRs) involves the cooperation of α, β, and γ subunits within the G protein complex. Upon GDP/GTP exchange on the Gα subunit, a structural reconfiguration takes place, causing the Gα and Gβγ subunits to separate during signal conduction. This allows the Gα subunit to freely interact with different binding partners for downstream signal transduction [22]. Guanine nucleotide-binding protein beta-4 (GNB4), a key component of heterotrimeric G protein composed of α, β, and γ subunits, coordinates cell proliferation, apoptosis, differentiation, and motility [23]. Elevated GNB4 protein expression is strongly indicative of a poor prognosis in various malignancies [24,25]. Elevated levels of GNB4 drive the activation of the Erk1/2 signaling pathway and cause a substantial increase in the expression of the key oncogenic transcription factor c-myc, thereby facilitating the proliferation and metastasis of gastric cancer (GC) cells [26]. Earlier research has indicated that certain small molecules may bind to Gβγ, consequently interfering with or facilitating the interaction between Gβγ and specific effectors [27]. So far, however, the relationship between circRNAs and Gβγ, particularly involving GNB4, remains uninvestigated.

Employing a circular RNA microarray, we examined circRNA expression profiles in BC tissues. Our investigation unveiled a novel circRNA originating from the RREB1 gene, denoted as circRREB1 (circBase ID: hsa_circ_0001573). Remarkably, circRREB1 displayed substantial upregulation in BC cells and tissues and demonstrated a close association with unfavorable clinical prognoses in BC patients. CircRREB1 was found to enhance the growth and metastatic potential of BC cells in both *in vivo* and *in vitro* assays. Subsequent mechanistic inquiries uncovered that circRREB1 facilitates BC progression through direct binding to GNB4, leading to the activation of Erk1/2 signaling pathways. It seems from our data that circRREB1 holds promise as both a therapeutic target and prognostic marker for BC.

2. Materials and methods

2.1. Human BC tissues and cell culture

Typically, 100 pairs of cancer and paracancerous specimens of surgically resected female BC patients were selected from the First Affiliated Hospital of Chongqing Medical University (China), and ahead of the surgical operation, every patient agreed to the procedure by signing an informed consent form. Tissue preservation in liquid nitrogen commenced immediately upon resection. The ethical approval for the work was obtained from the Ethics Committee of this same University. Normal human breast epithelial cells MCF-10A, BC cell lines MCF-7, SK-BR-3, and 293 T were preserved at our experimental research center, MDA-MB-231, MDA-MB-453, BT-549, and BT-474 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). MCF-10A, MCF-7, MDA-MB-231, and 293 T cells were grown in DMEM (Gibco, Carlsbad, CA, USA) comprising 20% FBS, SK-BR-3, MDA-MB-453, BT-549, and BT-474 were followingly cultured using RPMI 1640 medium (Gibco) containing 10% FBS. Cells were cultured at 37 °C in a 5% CO_2 environment, with medium renewal performed every 12–24 h.

2.2. RNA extraction, cytoplasmic-nuclear separation, and qRT-PCR analysis

Total RNA was initially extracted from MCF-10A cells, BC cells, BC tissues, and paracancerous tissues by Trizol protocols (Takara, Dalian, China). RNA integrity was identified by agarose gel electrophoresis, and its purity and concentration were detected with the Nanodrop 2000 (Thermo Fisher Scientific, USA). Cytoplasmic-nuclear separation was performed as per PARIS™ Kit (Thermo Fisher Scientific, USA) instructions. The total RNA was reversely transcribed into cDNA via the PrimeScript RT Reagent Kit (Takara). Then TB Green Premix Ex Taq (Takara) was adopted for qRT-PCR, and the reaction program was followed by 95 °C for 3 min, 95 °C for 5 s, 60 °C for 3 s, and 72 °C for 3 s. Three replicate amplifications were established for each sample. Relative expression of circRNAs and mRNAs was followingly quantified using the $2^{-\Delta\Delta Ct}$ and standardized to GAPDH levels for normalization. Supplemental Material 1: Table S1 displays a detailed list of primer sequences.

2.3. Actinomycin D treatment and RNase R digestion

BC cells logarithmically grown were equally seeded in 6-well plates (1 × 106 cells per well), and 100 ng/mL actinomycin D (Cell Signaling Technology, Beverly, MA, USA) was employed for BC cells treatment for 12h and 24h, respectively. Total RNA (1 μg) from BC cells was incubated at 37 °C with 3U/μg of RNase R (Epicentre Biotechnologies, Madison, WI, USA) for 10min, 20min, 30min, and 40min, respectively. The relative quantitative data of circRREB1 and its corresponding linear transcripts were detected by qRT-PCR.

2.4. Plasmid construction, siRNAs, and cell transfection

The circRREB1 sequence underwent amplification and subsequent cloning into the circRNA overexpression vector pLC5-ciR (Geneseed, Guangzhou, China). SiRNAs targeting the back splice site of circRREB1 (si-circ#1, si-circ#2, si-circ#3) and negative control were followingly synthesized by Geenseed. GNB4 overexpression vector was cloned from GNB4 complementary DNA (cDNA) into pcDNA 3.1–3 × Flag (RiboBio, Guangzhou, China), and the short-hairpin structure against GNB4 was designed and synthesized by RiboBio. The lentiviral overexpression vector LV-circRREB1 and knockdown sh-circRREB1 and the control vector LV-NC were provided by HanBio (shanghai, China), then MCF-7 cells were infected with them, respectively, and circRREB1-stable overexpression and control cell lines were obtained by puromycin screening and qRT-PCR identification. Following protocols, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was subjected to transfect all plasmids in subsequent experiments. Supplemental Material 1: Table S2 lists the sequences of siRNAs and shRNAs used.

2.5. Animal experiments

BALB/c nude mice, four weeks old and female, were obtained from Chongqing Tengxin Biotechnology Co, Ltd (Chongqing, China) for use in xenograft experiments and were housed in a pathogen-free facility. MCF-7 cells were subjected to transfection with either circRREB1 overexpression/mock vector or lentiviruses (Hanbio) carrying sh-circRREB1/sh-NC, and then 2×10^6 cells were injected subcutaneously into each nude mouse (six mice/group). The xenograft tumor growth was continuously monitored. Utilizing a vernier caliper, tumor volume (V) was computed from the length (L) and width (W) measurements, employing equation $V = (L \times W^2)/2$. Following a five-week interval, the mice were initially sacrificed, and their tumors were immediately harvested and weighed. After obtaining the lungs and livers for further investigation, which included HE staining and IHC, MCF-7 stable cells, as described previously, were introduced into the lateral vein of nude mouse tails. After four weeks, the mice were accordingly imaged with a small animal imaging system after gas anesthesia. Survival analysis was subsequently performed in parallel with the above tumor growth studies. All procedures adhered to the protocols approved by the Animal Care and Use Committee of Chongqing Medical University.

2.6. Fluorescence in situ hybridization (FISH) and immunofluorescence (IF) co-staining

The Cy3-labeled probe for circRREB1 was designed and synthesized by Geneseed. BC cells were notably inoculated in a 24-well plate with coverslips, and then the localization of circRREB1 in BC cells was detected using a FISH kit (RiboBio) abiding by the manufacturer's prescribed procedures. Later, BC cells were incubated with antibodies specific for GNB4 (1:50, Abcam, CA, USA) at 4 °C overnight and FITC-conjugated secondary antibodies at 37°Cfor 1.5 h, the co-localization of circRREB1 and GNB4 in BC cells was detected. Supplemental Material 3: Table S3 shows probe sequences.

2.7. Tissue microarrays (TMAs) and in situ hybridization (ISH)

Outdo Biotechnology (Shanghai, China) prepared TMA from 260 BCE tissues and 120 paracancerous tissues embedded in paraffin. A digoxin-labeled probe (Geneseed) was synthesized to detect the expression of circRREB1 with ISH. The TMAs were accordingly dewaxed in xylene and then rehydrated with 100, 90, 80, and 70% alcohol, then hybridized with the circRREB1 probe at 42 °C for 12h. The tissues were followingly incubated with a biotin-conjugated anti-digoxin antibody overnight at 4 °C, followed by NBT/BCIP. CircRREB1 expressions were quantified by multiplying the positive staining intensity score (negative = 0; weak = 1; medium = 2 and strong = 3) by the percentage of positive staining cells (0, <5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; 4, >75%). Sequences were detailed in Supplemental Material 1: Table S3.

2.8. Microarray analysis

Total RNA was extracted from postoperative postoperative invasive ductal carcinoma of the breast (patient 1: invasive ductal carcinoma, ER+, PR-, her-2+; patient 2/4: invasive ductal carcinoma, ER+, PR+, her-2+; and patient 3: invasive ductal carcinoma, ER-, PR-, her-2-) and paracancerous tissues of four female BC patients without radiotherapy treatment with Trizol reagent (Takara, Dalian, China) and accordingly quantified by the Nanodrop 2000 (Thermo Fisher Scientific, USA). Sample preparation and microarray hybridization procedures were carried out as per Arraystar's standard protocols by KangChen Bio-tech (Shanghai, China). Then differential circRNA expression profiles were obtained with P value < 0.05 and fold-change ≥ 2.

2.9. Cell cycle and apoptosis analysis

BC cells were plated in 6-well plates and then subjected to digestion using 0.25% trypsin, followed by collection with PBS buffer. Typically, the detection of apoptosis was accomplished through Annexin V/PI staining, with cell cycle distribution determined via flow cytometry. Evaluation of cell apoptosis involved Hoechst 33342 and TUNEL staining procedures as per the instructions furnished by Beyotime (Shanghai, China). Images were captured using a fluorescent microscope sourced from Leica (Wetzlar, Germany).

2.10. Cell proliferation wound healing, migration, and invasion assays

EdU, CCK-8, colony formation, wound healing, transwell migration, and invasion assays were executed according to previous studies [28].

2.11. Western blot analysis

Cells transfected for 48 h were harvested, followed by extraction of total protein and measurement of its concentration. The protein was denatured for 10 min in a metal bath at 100 °C and stored at −80 °C. Protein isolation was notably conducted through 10% SDS-PAGE, with subsequent transfer onto PVDF membranes (Bio-Rad, CA, USA). Membranes were exposed to 10% skimmed milk powder blocking for 2 h (Haimen Kylin-Bell Lab Instruments Co., Ltd, China); Primary antibodies against Caspase-3 (cleaved) (1:2000 dilution), Bcl-2 (1:1000), Bax (1:2000), GAPDH (1:5000), CCND1 (1:2500), CCNE1 (1:2000), CDK4 (1:2000), and GNB4 (1:1500) were then applied and accordingly incubated overnight at 4 °C. Subsequent incubation with secondary antibodies (1:5000) (Cell Signaling Technology) lasted for 1.5 h at 37 °C. Then the images were analyzed by chemiluminescence.

2.12. Immunohistochemistry (IHC)

IHC assays were executed according to previous studies [29].

2.13. RNA pull-down and RNA immunoprecipitation (RIP) assays

Synthesis of biotin-labeled probes aimed at the circRREB1 junction site was conducted by RiboBio. The assay was accordingly detected by Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific, USA) for RNA pull-down, as outlined by the manufacturer's protocols. The retrieved proteins were tested by Western blot and mass spectrometry analysis (Life Sciences Institute of Chongqing Medical University, China). Supplemental Material 3: Table S3 lists the sequences of the probe. RIP assays were conducted using an RNA immunoprecipitation kit (Geneseed) following the prescribed protocols to confirm the interaction between circRREB1 and GNB4. The circRREB1 and GNB4 expressions were measured by Western blot and qRT-PCR analysis. The negative control employed in the experiment was normal human IgG provided by Cell Signaling Technology. Supplemental Material 3: Table S1 shows the primer sequences used in our study.

2.14. Statistical analysis

SPSS 21.0 (IBM, SPSS, Chicago, IL, USA) and GraphPad Prism 8.0 (GraphPad Software Inc., CA, USA) were utilized for statistical analysis, with results presented as the mean ± standard deviation. A Student's t-test compared data between two groups, while one-way ANOVA analyzed data across multiple groups. The relevance between groups was subjected to the $\chi 2$ test. Interplays were examined through Pearson's correlation test, while the diagnostic efficacy of circRREB1 in BC was assessed via Receiver Operating Characteristic (ROC) curve analysis. Generally, survival curves were subjected to the Kaplan–Meier method, with comparison conducted through the log-rank test. Notably, multivariate Cox proportional hazards regression models were leveraged to identify factors affecting the overall survival of BC patients. $^*P$ value < 0.05 was statistically significant.

3. Results

3.1. CircRREB1 is identified and characterized in BC

Differential expression of circRNAs in BC was investigated using RNA microarray analysis on four patient samples of both tumor

and adjacent non-tumor tissues. Utilizing fold-change ≥2 and *P*-value <0.05 as cutoffs, we identified 112 circRNAs with significantly altered expressions. Among these, 90 were elevated and 22 were reduced (Supplemental Material 2). The heatmap shows 18 dysregulated circRNAs, eight of which were up-regulated. Among the significantly upregulated circular RNAs, circRREB1 (hsa_circ_0001573) stood out as a novel finding (Fig. 1A). As per circBase annotation, circRREB1 emerges from back-splicing of pre-mRNA, specifically spanning exons 2 to 6 of RREB1, measuring 709 bp in length. The back splicing junction of the RREB1 gene, found on human chromosome 6, was verified via Sanger sequencing (Fig. 1B). Verification of circRREB1's circular nature was conducted via RT-PCR, employing convergent and divergent primers, and utilizing GAPDH as an internal control. The findings highlighted that circRREB1 was exclusively identified in cDNA, indicating its absence in gDNA, while the convergent primers yielded amplification of circRREB1 from both cDNA and gDNA (Fig. 1C). Consequently, BC cells were subjected to treatment with actinomycin D at various time intervals, and qRT-PCR findings imply that the liner RREB1 mRNA transcript degraded rapidly, while the circRREB1 had stronger tolerance to actinomycin D (Fig. 1D). Moreover, the stability of circRREB1 was verified by RNase R digestion in BC cells, showing that circRREB1 showed a higher degree of resistance to RNase R digestion than RREB1. It can be inferred from the findings that the circular structure of circRREB1 confers greater stability compared to its linear transcripts (Fig. 1E). Subsequently, analysis of nuclear-cytoplasmic fractions alongside FISH assays demonstrated the localization of circRREB1 within both the nucleus and cytoplasm of BC cells. (Fig. 1F and G).

3.2. *CircRREB1 is expressed highly in BC tissues and associated with clinical characteristics*

In 100 pairs of BC and adjacent non-cancerous tissues, qRT-PCR was employed to assess the relative expression of circRREB1. The findings revealed a substantial upregulation of circRREB1 in BC tissues as opposed to their non-cancerous counterparts (Fig. 2A and B). The receiver operating characteristic (ROC) curve unraveled an area under the curve (AUC) of 0.707, suggesting that the circRREB1 expression serves as a superior diagnostic marker for distinguishing between tumor and non-tumor tissues. At a threshold of 25.135, the specificity and sensitivity were 47% and 92%, respectively (Fig. 2C). In our investigation involving 100 BCE patients, we explored the link between circRREB1 expressions and clinicopathological characteristics, as shown in Table 1. CircRREB1 expression exhibited a

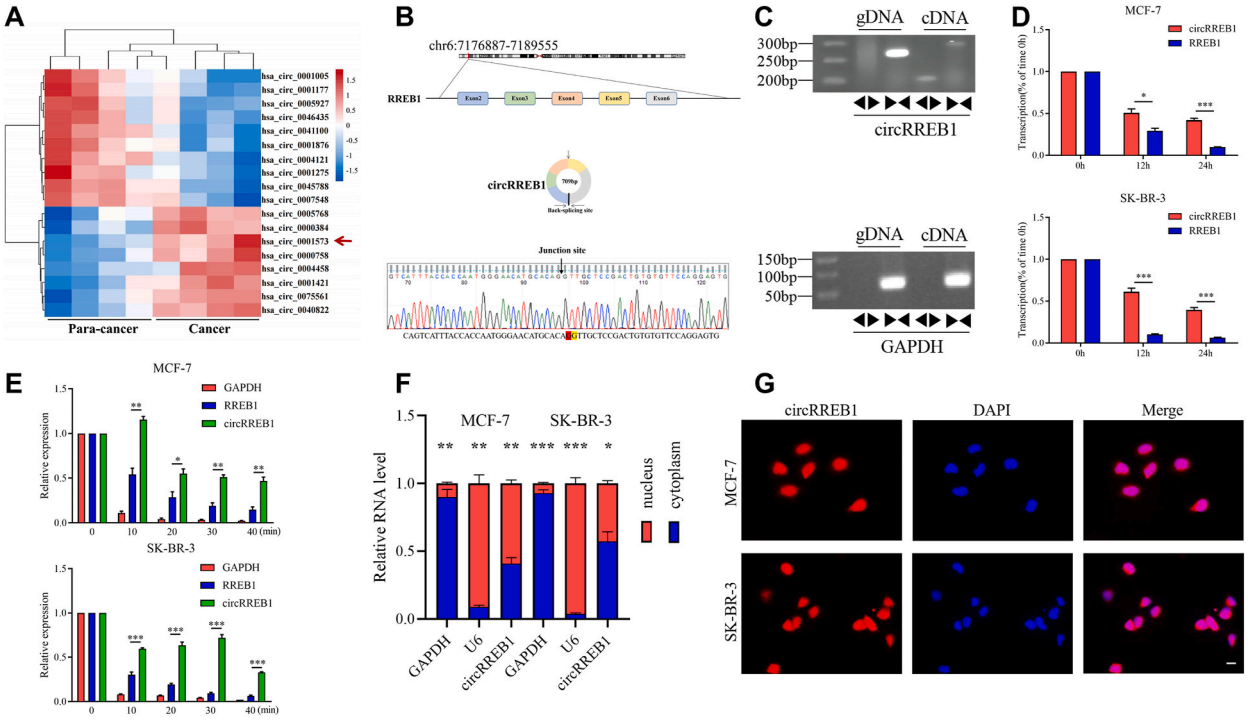

Fig. 1. CircRREB1 is validated and characterized in BC cells. A. Heatmap showed 18 differentially expressed circRNAs of microarray analysis in 4 pairs of human BC tissues and adjacent normal tissues. The red and blue strips indicate up-regulated and down-regulated circRNAs, respectively. B. The structure schematic of circRREB1: circRREB1 was generated from exon 2 to exon 6 of RREB1 by back-splicing. The back-splice junction sequences were confirmed by Sanger sequencing. C. The expression of circRREB1 in BC cells cDNA and gDNA was detected by qRT-PCR. D. The relative expression of circRREB1 and the corresponding linear transcript RREB1 of BC cells were detected by qRT-PCR after treatment with actinomycin D. E. The levels of circRREB1 and RREB1 were analyzed after RNase R digestion at different time points by qRT-PCR. F. Cytoplasmic-nuclear separation assay demonstrated that circRREB1 presented in both the nucleus and cytoplasm of BC cells. GAPDH was used as a positive reference for cytoplasmic localization and U6 was used as a positive reference for nuclear localization. G. The localization of circRREB1 was investigated in BC cells (magnification, × 400, Scale bar, 100um) with FISH. The data are presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

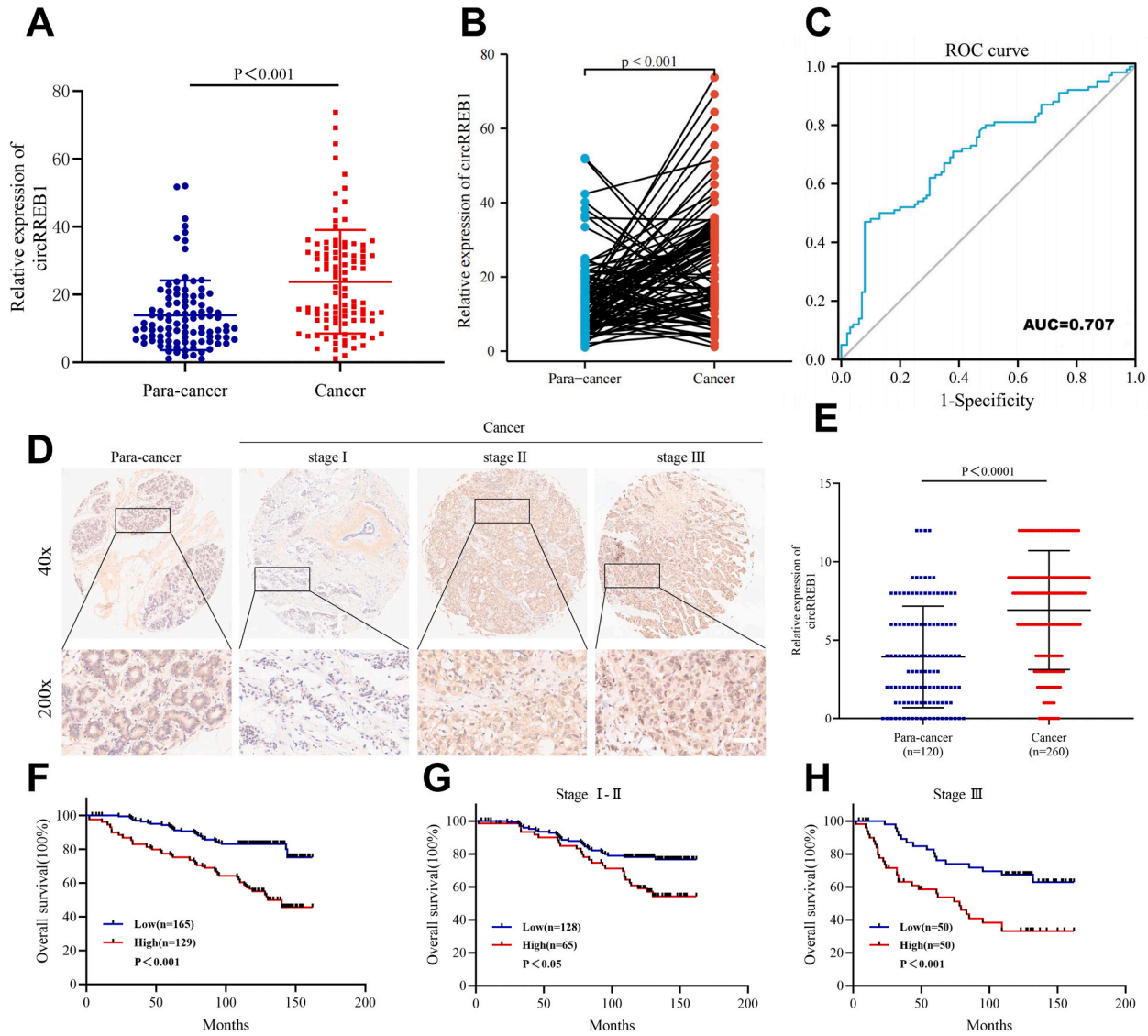

Fig. 2. CircRREB1 is significantly up-regulated in BC tissues and associated with poor prognosis in BC patients. A-B. Relative expression of circRREB1 in 100 pairs BC tissues and paracancerous tissues was detected by qRT-PCR. C. ROC curve was used to evaluate the diagnostic value of circRREB1. D. Representative images showed the expression of circRREB1 in 260 BCE tissues by ISH staining (magnification, × 200, Scale bar, 200um). E. Quantification of the ISH score of circRREB1 on TMAs. F–H. Kaplan-Meier survival curve analysis revealed that the survival time of BC patients with high expression of circRREB1 was significantly shorter than that of BC patients with low expression of circRREB1.

significant positive association with T ($P = 0.001$) and TNM ($P = 0.004$) stages, yet displayed no correlation with age, grade, or other clinicopathological parameters. To further determine circRREB1 expression in BC tissues, the human tissue microarrays (TAMs) containing paraffin-embedded 260 BCE tissues and 120 paracancerous tissues were used for ISH. The result showed that circRREB1 was upregulated in BC tissues (Fig. 2D and E). Table 2 outlines the correlation between circRREB1 expression and clinicopathological parameters in BC cases. Subsequently, Kaplan-Meier survival analysis indicated a shorter survival duration in patients with high circRREB1 expressions compared to those exhibiting low circRREB1 (Fig. 2F). Patients at stage I-II and stage III demonstrated analogous response patterns (Fig. 2G and H). Analysis using the Cox proportional hazard model demonstrated that elevated levels of circRREB1 were an independent indicator of unfavorable prognosis among BC patients (Table 3). In summary, elevated expression of circRREB1 is closely pertinent to poor prognosis for BC and might be a prognostic factor for BC patients.

3.3. CircRREB1 increases growth and regulates apoptosis of BC cells

CircRREB1 expression was examined across a panel of BC cell lines (MCF-7, SK-BR-3, MDA-MB-231, MDA-MB-453, BT-474, and BT-549) and MCF-10A cells, demonstrating a marked increase in circRREB1 levels specifically within MCF-7 and SK-BR-3 cells

Table 1
Correlation between circRREB1 expression and clinicopathological features in 100 BCE patients.

Clinicopathological features		All cases	Relative expression of circRREB1		Chi-square	*P* value
			low	high		
All cases		100	30	70		
Age	<54	79	19	60	6.341	0.012[a]
	≥54	21	11	10		
Grade	II	74	24	50	0.802	0.371
	III	26	6	20		
T stage	T1	25	14	11	10.730	0.001**
	T2-3	75	16	59		
N stage	N0	44	16	28	1.516	0.218
	N1-3	56	14	42		
TNM stage	I	17	10	7	8.103	0.004**
	II/III	83	20	63		

[a] $P < 0.05$, $^{**}P < 0.01$.

Table 2
Correlation between circRREB1 expression and clinicopathological features in 260 BCE patients.

Characteristic		All cases	CircRREB1		Chi-square	*P* value
			low	high		
All cases		260	136	124		
Age	<54	136	64	72	3.145	0.076
	≥54	124	72	52		
Grade	II	193	97	96	1.260	0.262
	III	67	39	28		
T stage	T1	79	54	25	11.713	<0.001***
	T2-3	181	82	99		
N stage	N0-1	120	73	47	6.493	0.011*
	N2-3	140	63	77		
TNM stage	I	39	32	7	16.271	<0.001***
	II/III	221	104	117		

$^{*}P < 0.05$, $^{***}P < 0.001$.

Table 3
Univariate and multivariate Cox regression analysis of circRREB1 and survival in 247 BCE patients.

Clinical variables	Univariate analysis		*P*	Multivariate analysis		*P*
	HR	95%CI		HR	95%CI	
Age (≥54 vs. <54)	1.01	0.99–1.03	0.225			
Grade (II vs. III)	1.96	1.24–3.09	0.005**	2.22	1.40–3.53	0.001**
T stage (T1 vs. T2/3)	3.16	1.63–6.14	<0.001***	2.94	1.38–6.25	0.005**
N stage (N0-1 vs. N2-3)	5.92	1.45–24.12	0.013*	1.19	0.23–6.11	0.835
TNM stage (I vs. II/III)	1.96	1.22–3.15	0.005**	2.06	1.25–3.42	0.005**
CircRREB1 (low vs. high)	2.11	1.28–3.49	0.004**	1.74	1.04–2.91	0.033*

Abbreviations: HR hazard ratio, CI confidence interval, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

(Fig. 3A). Additionally, circRREB1 overexpression plasmids and siRNAs were synthesized and transfected into BC cells. Subsequent analysis revealed a significant increase or decrease in circRREB1 expression in BC cells with designated vectors or siRNAs, as determined by qRT-PCR (Fig. 3B and C), while linear transcripts remained unaffected (Supplemental Material 3: Fig. S1A). Increased expression of circRREB1 notably augmented BC cell proliferation, as demonstrated by colony formation, EdU, and CCK8 assays, while depletion of circRREB1 exhibited contrasting outcomes. (Fig. 3D–H). Staining with Hoechst 33342 indicated that BC cells in the si-circ group exhibited evident apoptotic features such as nuclear fragmentation and intense fluorescence. TUNEL assay further confirmed a rise in apoptotic cell count compared to control groups following circRREB1 knockdown (Fig. 3I and J). Downregulation of circRREB1 markedly enhanced apoptosis in BC cells, as determined by Annexin V/PI flow cytometry (Fig. 3K–L). Finally, the si-circ group displayed reduced Bcl-2 protein expression and significantly heightened levels of Bax and cleaved Caspase-3 compared to the si-NC group, as observed in the Western blot results (Fig. 3M − N).

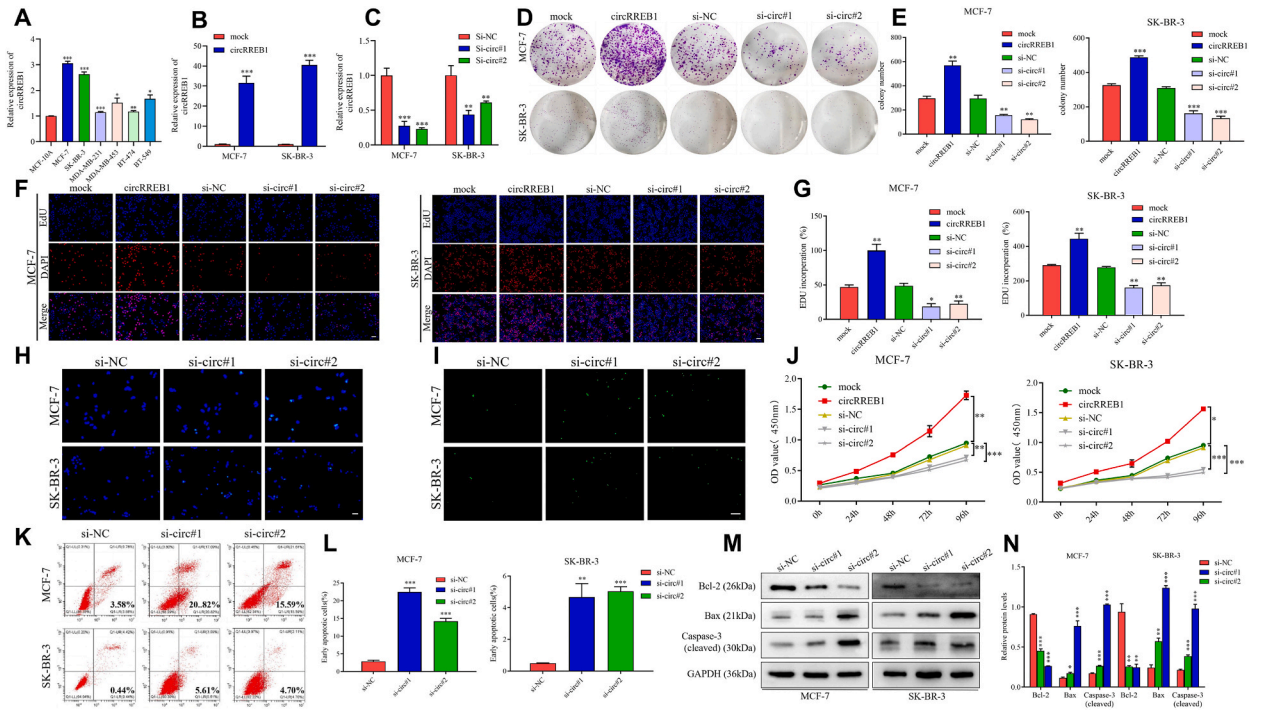

Fig. 3. CircRREB1 enhances proliferation and suppresses apoptosis of BC cells. A. The relative expression of circRREB1 in MCF-10A cells and BC cells was measured by qRT-PCR. B–C. Overexpression efficiency of circRREB1 and the effect of si-circRREB1 were validated by qRT-PCR. D-E. Colony formation assay was used to detect the effect of overexpression and knockdown of circRREB1 on the survival ability in BC cells. F-G. EdU assay was conducted to detect the cell proliferation ability of BC cells (magnification, × 100, Scale bar, 0.5 mm). H. CCK-8 assay was used to value cell viability. I. The apoptotic morphological features of BC cells transfected with siRNAs were revealed by Hoechst 33342 (magnification, × 400, Scale bar, 200um). J. Apoptotic cells were displayed after the circRREB1 knockdown with TUNEL staining (magnification, × 100, Scale bar, 0.5 mm). K-L. The apoptotic rate was examined by flow cytometry using AnnexinV/PI-staining after knockdown circRREB1. M-N. The expression levels of related apoptotic proteins were detected in BC cells transfected with indicated siRNAs by Western blot. The data are presented as the mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.4. CircRREB1 enhances migration, and invasion and regulates the cell cycle of BC cells

The migratory and invasive potentials of BC cells were notably augmented upon circRREB1 overexpression but substantially attenuated upon circRREB1 knockdown, as determined by wound healing and transwell assays (Fig. 4A–F). Flow cytometry assay results showed that reduction of circRREB1 expression resulted in a substantial rise in G1 phase cell population alongside a decrease in S and G2 phases, indicating G1 phase cell cycle arrest induced by si-circRREB1 in BC cells (Fig. 4G and H). Moreover, Western blot analysis indicated a significant decrease in cell cycle-related proteins CCND1, CCNE1, and CDK4 expressions upon circRREB1 knockdown in BC cells (Fig. 4I and J).

3.5. CircRREB1 directly binds to GNB4 and GNB4 increases p-Erk1/2 expression

CircRREB1-specific probes were designed, and RNA pull-down assay, mass spectrometry, and further bioinformatics analysis results showed that GNB4 was found to be significantly enriched on circRREB1 (Fig. 5A and B), and a unique peptide was identified by mass spectrometry analysis (Supplemental Material 3: Fig. S1B). RIP assay unraveled that the enrichment of circRREB1 immunoprecipitated with GNB4 antibody was significantly increased compared to the negative control IgG (Fig. 5C). FISH and IF co-staining assays showed that circRREB1 and GNB4 were co-localized in the MCF-7 and SK-BR-3 cells, providing additional evidence for their interaction (Fig. 5D). KEGG pathway analysis of proteins identified by mass spectrometry after RNA pull-down was closely correlated with the Erk1/2 signaling pathway (Fig. 5E). Therefore, GNB4 regulatory role was investigated in Erk1/2 signaling after GNB4 overexpression and knockdown, respectively. Western blot results showed that *p*-Erk1/2 expressions were dramatically elevated in the overexpression group relative to the mock group, suggesting that Erk1/2 signaling was activated, whereas Erk1/2 signaling was inhibited after GNB4 knockdown (Fig. 5F and G). Additionally, in 20 clinical BC samples. qRT-PCR results implied that GNB4 expressions in cancerous tissues were significantly higher than those in paracancerous tissues (Fig. 5H). Pearson correlation analysis indicated a strong positive correlation between circRREB1 levels and GNB4 expression (Fig. 5I).

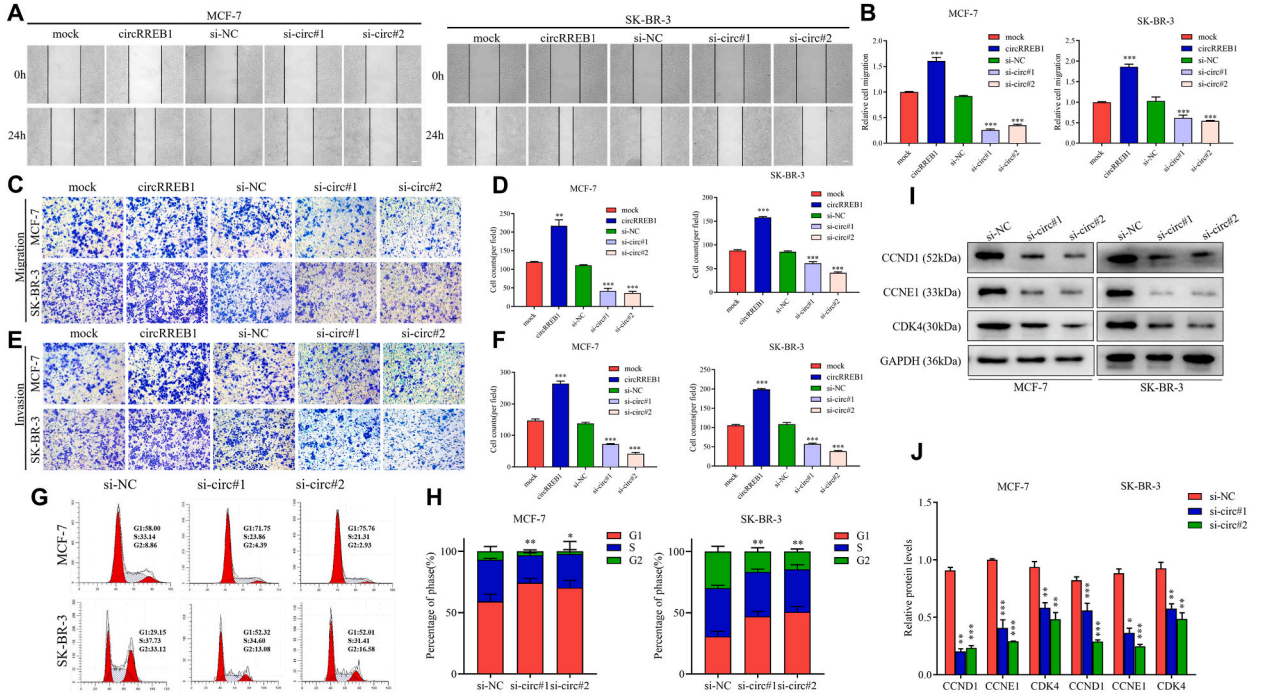

Fig. 4. CircRREB1 increases migration and invasion and modulates the cell cycle progression of BC cells. A-B. The migration ability of BC cells was detected by wound healing assay (magnification, × 50, Scale bar, 1 mm) after overexpression or knockdown of circRREB1. C–F. Transwell assays were performed to explore the migration and invasion ability of BC cells transfected with circRREB1 overexpression plasmid and si-circRREB1 (magnification, × 100, Scale bar, 0.5 mm). G-H. Cell cycle analysis was implemented by flow cytometry after circRREB1 silencing. I-J. The expression levels of cycle-related proteins were detected in BC cells transfected with indicated siRNAs by Western blot. The data are presented as the mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.6. Depletion or upregulation of GNB4 reverses circRREB1 overexpression or knockdown-induced biological functions

Building upon the aforementioned findings, rescue experiments were undertaken to delve deeper into the potential of circRREB1 in facilitating the migration, invasion, and proliferation of BC cells via its interaction with GNB4. CircRREB1 and sh-GNB4 co-transfection or si-circ#1 and OE-GNB4 co-transfection were performed to assess proliferation (CCK8, EdU assays) and migration/invasion (transwell assays) in BC cells. Results exhibited that changes in GNB4 levels significantly countered the effects of circRREB1 down-regulation and upregulation on the biological functionalities of BC cells (Fig. 6A–G). The application of Western blot allowed for the investigation into the detailed molecular mechanisms governing the interaction between circRREB1 and GNB4. CircRREB1 over-expression induced higher levels of *p*-Erk1/2 and c-Myc expressions in MCF-7 cells compared to the mock group, while c-Jun or c-Fos levels remained unchanged. However, this effect was reversed by downregulating GNB4. Furthermore, as against the si-NC group, the expressions of *p*-Erk1/2 and c-Myc in SK-BR-3 cells remarkably declined after circRREB1 silencing, but the upregulation of GNB4 could restore this effect. These data suggest that the interaction between circRREB1 with GNB4 could further activate Erk1/2 signaling (Fig. 6H and I).

3.7. CircRREB1 promotes tumorigenesis, and metastasis of xenograft tumors in vivo

To elucidate the biological functions of circRREB1 *in vivo*, MCF-7 cells were exposed to lentiviral infection with constructs for circRREB1 overexpression or knockdown, as well as their respective controls. Following that, the cells with stable infection were introduced into female nude mice aged four weeks. Within the subcutaneous xenograft tumor nude mice model, circRREB1 over-expression significantly elevated both the volume and weight of xenograft tumors relative to the control group, demonstrating a pronounced promotion of tumor growth. Conversely, circRREB1 knockdown attenuated xenograft tumor growth (Fig. 7A–C). We found that increased expression of circRREB1 induced spontaneous lung metastases in nude mice, demonstrated by a notable elevation in the number of metastatic pulmonary nodules as against the control group, and a marked reduction in nodular incidence in the circRREB1 knockdown cohort (Fig. 7D and E). Furthermore, there was a higher density of tumor microvessels observed in the circRREB1 overexpression group as opposed to the control group. Meanwhile, tumor angiogenesis exhibited a significant decrease in the silence group (Fig. 7F and G). In the tail vein of the BALB/c nude mice model, tumor metastasis in mice was detected by bioluminescence imaging (BLI). The findings indicated that the circRREB1 overexpression group exhibited heightened bioluminescence signals, while the silencing of circRREB1 resulted in diminished signals (Fig. 7H). The circRREB1 overexpression group demonstrated

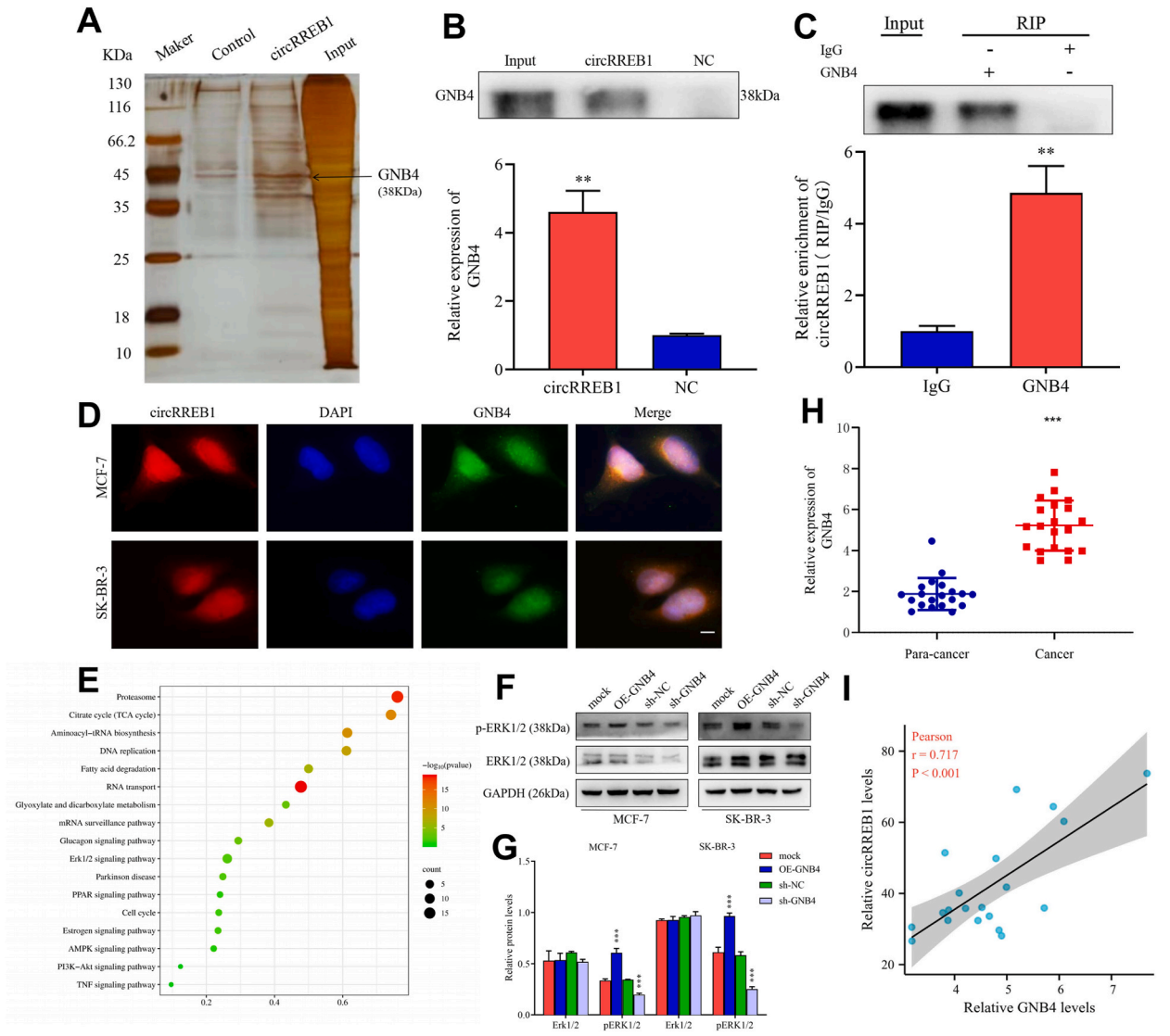

Fig. 5. CircRREB1 binds to GNB4 and GNB4 enhances the protein level of *p*-Erk1/2 in BC cells. A. RNA pull-down assay was performed using specific biotin-labeled circRREB1 to detect the proteins interacting with circRREB1, mass spectrometry and silver staining were performed to identify the interacting proteins. B. The interaction between circRREB1 and GNB4 was verified by Western blot. C. The enrichment ratio of circRREB1 with GNB4 antibody was determined by RIP assay, compared with IgG. D. The co-localization of circRREB1 and GNBE in BC cells was examined by FISH and IF co-staining (magnification, × 400, Scale bar, 50um). E. KEGG pathway analysis of pulled-down proteins from BC cells with biotin-labeled probes targeting the circRREB1. F-G. The expression of Erk1/2 and *p*-Erk1/2 was detected by Western blot after GNB4 overexpression or knockdown. H. The relative expression of GNB4 in 20 pairs of BC tissues and paracancerous tissues was detected by qRT-PCR. I. Pearson correlation analysis revealed that the expression of GNB4 was positively correlated with the level of circRREB1. The data are presented as the mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

inferior overall survival time in mice, accompanied by an increased incidence of liver metastatic nodules and larger nodular size in the liver, as compared to the control group (Fig. 7I and J). Finally, to detect the effect of circRREB1 on the expressions of cycle-related proteins, ki67 and *p*-Erk1/2 by IHC. When circRREB1 was overexpressed, there was a noticeable increase in the expression levels of CCND2, CDK4, ki67, and *p*-Erk1/2 as against the control group. Conversely, silencing circRREB1 resulted in reduced expression of these proteins (Fig. 7K). In brief, the data outlined above indicates that circRREB1 might boost tumorigenesis and metastasis in nude mice, suggesting its oncogenic involvement in BC progression.

4. Discussion

The burgeoning landscape of circular RNAs (circRNAs) continues to unravel their profound implications in various biological

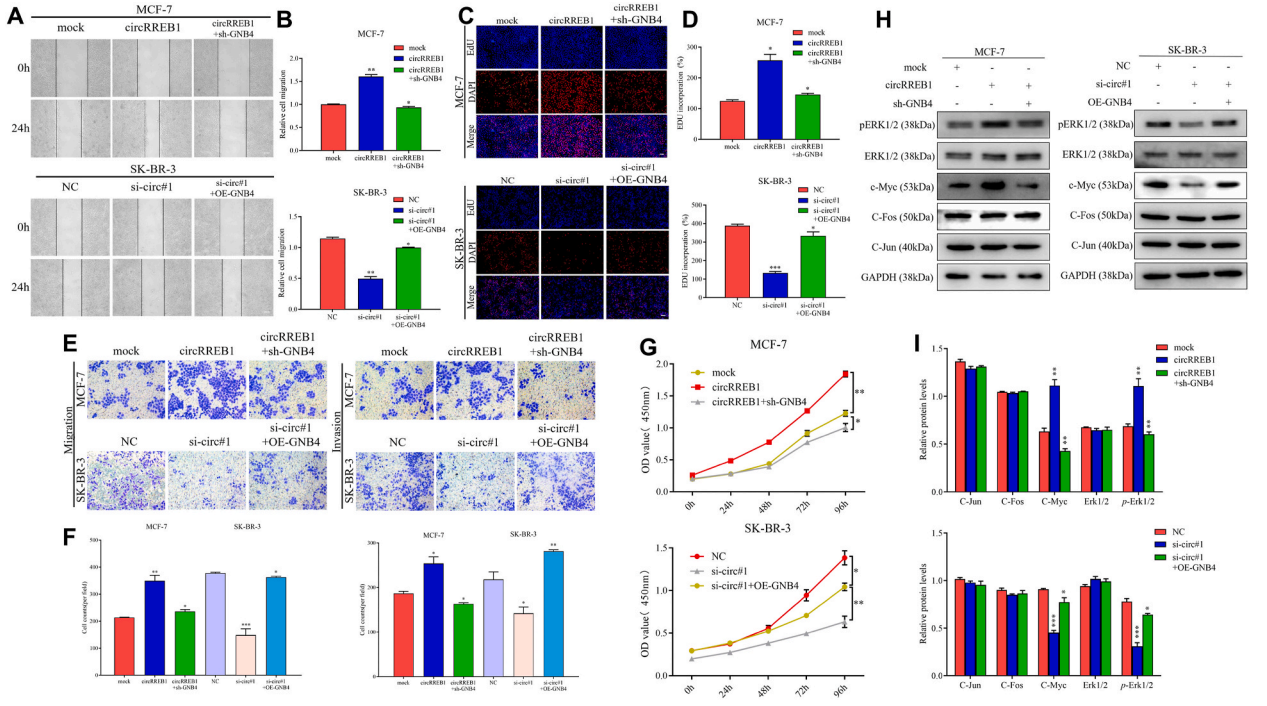

Fig. 6. Downregulation or overexpression of GNB4 could restore the abilities of proliferation, migration, invasion of BC cells, and *p*-Erk1/2 expression by upregulating or silencing circRREB1. A-B. Wound healing assay was performed to determine the migration ability of BC cells after transfected with circRREB1 plasmid or si-circ#1 or co-transfected with indicated vectors with sh-GNB4 or OE-GNB4 (magnification, × 50, Scale bar, 1 mm). C-D. EdU assay was utilized to analyze the effect of GNB4 and circRREB1 interactions on the proliferation capacity of BC cells. (magnification, × 100, Scale bar, 0.5 mm). E-G. Transwell migration and transwell invasion assays (magnification, × 100, Scale bar, 0.5 mm) or CCK8 assays were executed to detect the effect of GNB4 and circRREB1 interactions on the migration and invasion ability of BC cells or cell viability. H–I. The effect of interaction between GNB4 and circRREB1 on the expression of key factors of the Erk1/2 signaling pathway was detected by Western blot. The data are presented as the mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

processes, particularly in the intricate realm of tumorigenesis and metastasis [30,31]. Despite the increased focus on identifying and characterizing circRNAs, there remains a substantial lack of understanding, with the biological functions and underlying mechanisms of the majority of circRNAs remaining enigmatic. In the context of BC, our study sought to unravel a novel circRNA, circRREB1, which is significantly up-regulated in BC. Increased circRREB1 expression was observed to be significantly linked with poorer prognostic outcomes in BC patients. Functional studies exhibited that circRREB1 markedly boosted BC proliferation, migration, and metastasis both *in vivo* and *in vitro*. Mechanistically, we found that circRREB1 could directly combine with GNB4 to further activate the Erk1/2 signaling, thus promoting BC progression.

Generally, many circular RNAs function as miRNA sponges, exerting regulatory control over gene expression [32]. However, emerging evidence shows that circRNAs can influence the development of some tumors by interacting with proteins and modulating their functions [33]. Circular RNAs have been identified as regulators that sequester microRNAs, interact with key proteins like p53, VEGF, or c-myc within critical tumor-suppressive and pro-oncogenic networks, and modulate the expression of gene clusters while also encoding peptides across diverse cancer cell types [34]. They can also interact with RBPs and regulate transcription, participating in many pathological processes, especially cancer metastasis [35]. CircRNAs have been noted for their ability to encode proteins/peptides involved in driving cancer pathogenesis and progression [36]. For example, circCD44 promotes TNBC invasion, proliferation, migration, and tumorigenesis partially through directly binding to IGF2BP2 [37]. Furthermore, circMYBL2 facilitates FLT3 translation through PTBP1 recruitment, contributing to FLT3-ITD AML development [38]. Additionally, circ-SIRT1 promotes growth, invasion, and EMT of CRC cells via binding to EIF4A3 and recruiting EIF4A3 [39]. Here, through RNA pull-down, mass spectrometry, and RIP assays, our results establish that circRREB1 binds to GNB4, underscoring the importance of circRNA-protein interactions in tumorigenesis.

Within the G protein-coupled receptor (GPCR) pathway, GNB4 assumes a central role by acting as a major component of heterotrimeric G proteins, facilitating the transmission of signals from cell surface receptors to internal effectors [40]. Several studies have highlighted the strong association of GPCRs with various aspects of cancer progression, including tumor growth, metastasis, and survival [41]. GNB4 overexpression is strongly pertinent to the poor prognosis in various cancers including gastric cancer, bladder cancer, and CRC [42–44]. GNB4 was determined to be an independent prognostic factor in individuals with H. pylori-induced GC [45]. It has been established that GNB4 is implicated in the progression and chemoresistance of BC [24]. Another study reported that GNB4 inhibits the growth of both antiestrogen-resistant and sensitive BC cells. Our findings consistently reveal heightened expression of

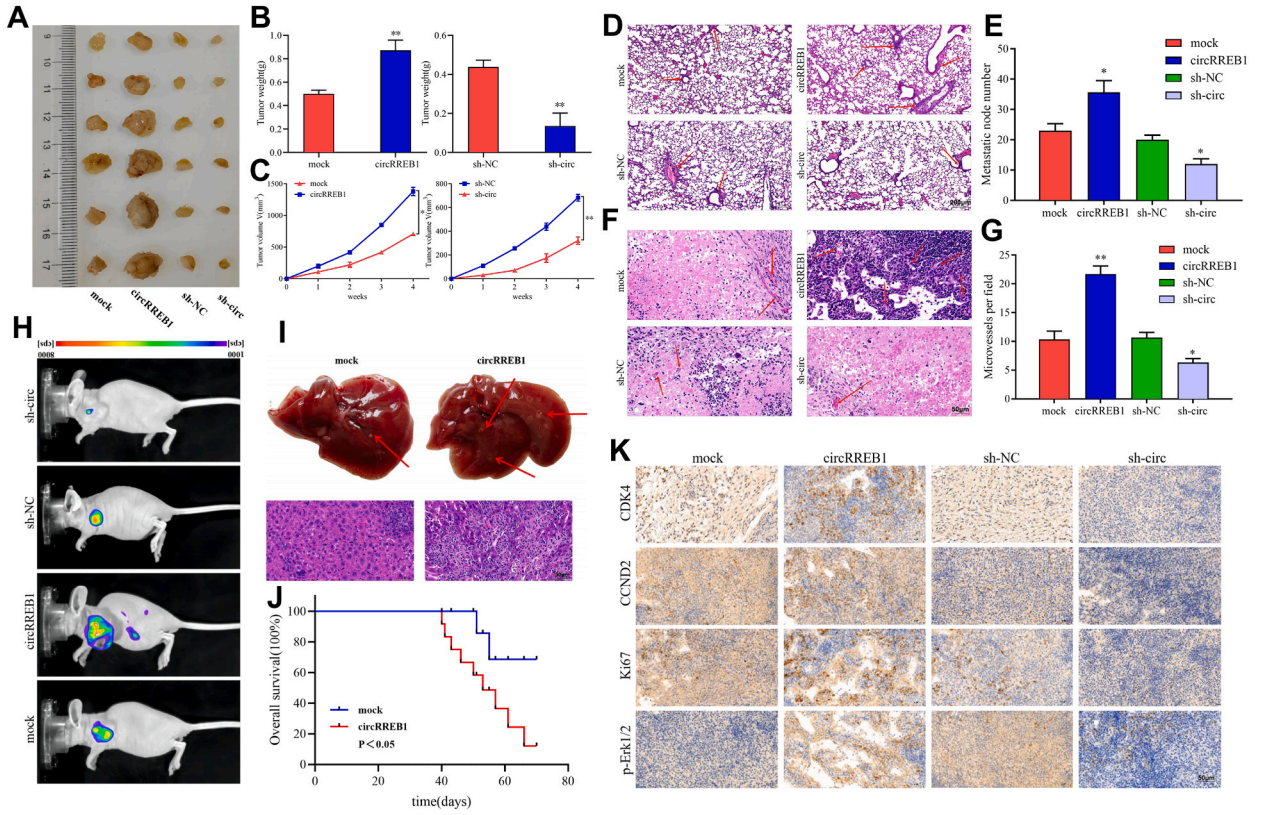

Fig. 7. CircRREB1 promotes tumorigenesis, and metastasis of xenograft tumors *in vivo*. A-B. Representative images of xenograft tumors and tumor weight of each group. C. The tumor growth curve was drawn by measuring the length and width of the tumor every week with a vernier caliper. D-G. Metastatic nodules of the lungs and microvessels of the tumors were analyzed by HE staining (magnification, × 100, Scale bar, 200um). H. Representative bioluminescent imaging (BLI) of the different groups was displayed. I. The representative images of the liver and HE staining showed pulmonary metastasis in the metastatic xenograft model by tail vein injection (magnification, × 400, Scale bar, 50um). J. The survival curve was plotted for the tumor-bearing mice using Kaplan–Meier survival analysis. K. The expressions of *p*-Erk1/2, CDK4, CCND2, and Ki67 in tumors of nude mice were detected by ISH (magnification, × 400, Scale bar, 50um). The data are presented as the mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

GNB4 in BC tissues, accompanied by a positive correlation between circRREB1 and GNB4 expression. These insights offer new avenues for understanding the regulatory role of GNB4 in BC growth [23].

Notably, a previous study has shown that GNB4 can activate the Erk1/2 signaling pathway, thereby facilitating the cell proliferation and metastasis of GC cells, which might be an underlying therapeutic target for GC [26]. The initiation and advancement of diverse cancer types, including BC, involve the activation of Erk1/2 signaling [46–48]. Gβγ dimers regulate many effectors including phospholipases, adenylyl cyclases (AC), ion channels, G protein-coupled receptor kinases (GRKs), β-adrenergic receptor kinase (βARK), and PI3Ks. Hence, Gβγ complexes have the potential to influence cellular signaling pathways linked to proliferation and differentiation by activating the Ras/ERK and PI3K/AKT pathways [49]. It has been demonstrated that G-protein-mediated signaling pathways can lead to ERK activation. Protein kinase A (PKA) and protein kinase C (PKC) are vital members of G-protein-dependent signaling pathways. G-protein-dependent activation of ERK1/2 can be abolished in cells of pretreatment with the PKA inhibitor H89 and the PKC inhibitor GF109203X [50]. The activation of ERK cascades through G-protein α subunit and G-protein βγ subunits signaling to Ras has been established. For instance, it has been reported that the Erk1/2 cascades could be activated through G-protein βγ subunit signaling to Ras-MAPK signaling [51]. The activated Erk1/2 signaling causes different downstream signal cascades. Activated ERK1/2 undergoes translocation to the cell nucleus, initiating phosphorylation of various transcription factors such as c-MYC, FOS, FRA1, and EGR1. Notably, these downstream effectors modulated by ERK1/2 then modulate a plethora of cellular processes, including proliferation, differentiation, and transformation [52]. For example, the transcription of MYC could be activated through the circACTN4/FUBP1/FIR axis, MYC serves as a regulatory subunit for the expressions of CCND2 and CDK4 to promote cell-cycle progression, whose activity is key for cell cycle G1/S transition, thus promoting the development of BC [53]. Additionally, research has demonstrated that the interaction of circCDYL2 with GRB7 and FAK could maintain the activity of downstream Erk1/2 signaling, thereby promoting the progression of HER2+ BC [54]. The overexpression of hsa_circ_0000190 induced the phosphorylation of Erk1/2, proliferation, and promoted migration and the growth of xenograft tumor [55]. More importantly, a study demonstrated that some small molecules regulated Gβγ-effector interactions by binding small molecules to Gβγ [24]. We found that circRREB1 up-regulation could significantly elevate the levels of *p*-Erk1/2 and c-Myc, a key nuclear transcription factor downstream of Erk1/2,

the knockdown of GNB4 could counteract this effect. Furthermore, the *p*-Erk1/2 and c-Myc expressions were decreased after circRREB1 silencing, but the overexpression of GNB4 could recover this effect. Therefore, we speculate that circRREB1 might enhance the Gβγ-effector interactions by combining with GNB4, further activating the Erk1/2 signaling. However, detailed mechanisms in the interaction between circRREB1 and GNB4 still need to be further explored.

Certain shortcomings are evident in this study. First, in the context of cancer, classical epithelial-mesenchymal transition proteins feature prominently in driving tumor progression and metastasis, which is a complex cellular program that enables epithelial cells to undergo a phenotypic transformation, acquiring mesenchymal traits. This change is associated with increased cellular motility, invasiveness, and resistance to apoptosis, all vital for cancer cells to migrate and initiate secondary tumor growth in distant locales. The EMT proteins were not detected in the current study, this point will be a valuable research field to explore the interplay between circRREB1 and classical EMT proteins in our future study. Besides, this study demonstrated the direct interaction between circRREB1 and GNB4, and GNB4's regulatory role in circRREB1 expression or biological functions, while whether circRREB1 could affect the level of GNB4 remains elusive, further study focusing on this point is needed.

5. Conclusion

Taken together, our results demonstrate a pronounced overexpression of circRNA circRREB1 in BC, which is indicative of a poor prognosis. Moreover, the findings implied that circRREB1 has the potential to act as an oncogenic factor in BC advancement. Mechanistically, our findings indicate that circRREB1 functions by directly engaging with GNB4, resulting in the activation of Erk1/2 signaling, ultimately driving the progression of BC. Our data reveal that circRREB1 might be a promising BC therapeutic target and prognostic biomarker.

Funding statement

This work was supported by grants from the National Natural Science Foundation of China (82173170).

Data availability statement

The datasets mentioned in this manuscript were available in circBase (http://circrna.org/), and the UCSC Genome Browser (http://genome.ucsc. edu/).

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (2022-K228; June 10, 2022). Human tissue study was approved by the Ethics Committee of Chongqing Medical University.

Consent for publication

Not Applicable.

CRediT authorship contribution statement

Hong Chen: Writing – original draft, Investigation, Data curation. **Xiaosong Wang:** Methodology. **Hang Cheng:** Methodology. **Yumei Deng:** Data curation. **Junxia Chen:** Supervision, Conceptualization. **Bin Wang:** Writing – review & editing, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank the Molecular Medicine and Cancer Research Center of Chongqing Medical University for providing us with materials to conduct experiments in the present study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28785.

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