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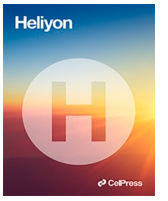

Research article

CircACTR2 promotes bladder cancer progression through IKBKB-mediated NF-κB signaling pathway activation

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ABSTRACT

Background: Circular RNAs (circRNAs) have significant roles in tumor progression. The role of circRNA derived from ARP2 actin-related protein 2 homolog (circACTR2) has been reported in various human diseases. However, the functions and regulatory mechanisms of circACTR2 in Bladder Cancer (BCa) remain unknown.
Objectives: This study aims to explore the biological role and regulatory mechanism of circACTR2 in BCa.
Methods: We analyzed the effects of circACTR2 on BCa through bioinformatics analyses, RT-qPCR, and cell function assays.
Results: We observed the upregulation of circACTR2 in BCa tissues and validated its circular structure. Loss-of-function assays demonstrated that silencing circACTR2 suppressed the proliferation, invasion, and migration of BCa cells. Mechanistic investigation revealed that circACTR2 sponges miR-219a-2-3p to elevate the expression of the inhibitor of nuclear factor kappa B kinase subunit beta (IKBKB). This induced upregulation of IKKβ protein promoted the nuclear translocation of p65, thereby activating the NF-κB signaling pathway.
Conclusions: Our findings indicate that circACTR2 promotes BCa cell proliferation, migration, and invasion by activating the NF-κB signaling pathway via the miR-219a-2-3p/IKBKB axis, potentially unveiling a new therapeutic target for BCa.

1. Introduction

Bladder cancer (BCa) is a highly prevalent malignancy originating from the urinary tract [1]. According to the 2022 American Cancer Society's estimates for BCa, there are approximately 81,180 new cases and 17,100 deaths in the United States. Occupational

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Abbreviation

BCa	Bladder cancer
circRNA	Circular RNA
miRNA	microRNA
ACTR2	Actin Related Protein 2
siRNA	Small interfering RNA
CCK-8	Cell Counting Kit-8
FISH	Fluorescence in situ hybridization
CeRNA	Competing endogenous RNA

exposure to chlorate hydrocarbons, polycyclic aromatic hydrocarbons, and aromatic amines, are strongly linked to BCa [2]. Advances in molecular biology associated with tumorigenesis are conducive to developing novel therapeutic method for BCa [1]. In this study, we intend to unveil the novel molecular mechanism underlying BCa progression.

Circular RNA (circRNA) features a covalently closed loop structure, conferring high stability [3]. CircRNAs play critical functions in various biological processes by acting as microRNA (miRNA) sponges and protein templates [4]. As research on BCa-related molecular mechanisms advances, an increasing number of dysregulated circRNAs have been identified as potential oncogenes [5–7]. Hsa_circRNA_102747 (855 nt; circBase ID: hsa_circ_0008529), derived from Actin Related Protein 2 (ACTR2), is a novel circRNA and termed circACTR2 in this report. Analysis of GSE147984 datasets revealed that circACTR2 was aberrantly upregulated in BCa tissues compared to matched non-tumor tissues, indicating a potential role for circACTR2 in BCa progression. Previous studies have demonstrated the modulatory function of circACTR2 in the fibrosis of renal tubular cells [8], M2 polarized macrophages-related renal fibrosis [9], and the dysfunction of human renal mesangial cells in diabetic nephropathy [10]. However, whether circACTR2 can modulate BCa cell functions is unknown. The present study aims to unveil the relevance between circACTR2 and BCa cell functions, potentially deepening the understanding of BCa progression-associated molecular mechanisms.

2. Materials and methods

2.1. Clinical samples

Paired samples (tumor and adjacent normal) were isolated from 50 patients who were diagnosed with BCa and underwent surgery at Jinling Hospital, Jinling School of Clinical Medicine, Nanjing Medical University. All patients were initially diagnosed with bladder cancer and met the surgical indications. Before surgical resection, all patients were not treated with chemotherapy, radiotherapy, or any other kinds of adjuvant therapy. After the collection, all tissue samples were immediately frozen and stored at − 80 °C. Samples were confirmed by two pathological experts. Before tissue collection, all patients or their guardians signed the informed consent. The clinical study was approved by the Ethical Committee of Jinling Hospital, Jinling School of Clinical Medicine, Nanjing Medical University (20200603114).

2.2. Cell lines

The human uroepithelial cell line (SV-HUC-1) and the human embryonic kidney HEK293T cell line were procured from ATCC (Manassas, VA, USA). Human BCa cell lines (T24, J82, 5637, and TCCSUP) were provided by Process (Wuhan, China). The T24 cell was maintained in McCoy's 5A. J82 and TCCSUP cell lines were cultured in MEM supplemented with NEAA. The 5637 cell line was maintained in RPMI 1640. The SV-HUC-1 cell line was cultured in Ham's F-12K. The HEK293T cell line was maintained in DMEM. Culture mediums for all cell lines were supplemented with 10 % FBS and 1 % Penicillin/Streptomycin. All cell lines were incubated in a humidified atmosphere with 5 % CO_2 at 37 °C.

2.3. Transfections

Small interfering RNAs (siRNAs) targeting circACTR2 (si-circACTR2-1, si-circACTR2-2) and a negative control siRNA (si-NC) were synthesized by Ribobio (Guangzhou, China). For IKBKB overexpression, the entire sequence of IKBKB was sub-cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) to generate the pcDNA3.1/IKBKB expression vector; the empty pcDNA3.1 vector was used as the negative control (NC). Overexpression or knockdown of miR-219a-2-3p was achieved using miR-219a-2-3p mimics and inhibitors synthesized by Ribobio, with corresponding NCs as controls. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, achieving a transfection success rate of approximately 80 %. The sequences involved in these experiments are detailed in Table 1.

Table 1
Sequences used in transfections.

si-NC	AAAAACTTTCTGATCTTATGG
si-circACTR2-1	TTTTTGAAAGACTAGAATACC
si-circACTR2-2	CCTTTTTGAAAGACTAGAATA
mimics NC	uucuccgaacgugucacguttacgugacacguucggagaatt
miR-219a-2-3p mimics	agaauuguggcuggacaucuguggcugagcuc
inhibitor-NC	uucuccgaacgugucacguttacgugacacguucggagaatt
miR-219a-2-3p inhibitor	gagcucagccacagauguccagccacaauucu
pcDNA3.1	none
pcDNA3.1/IKBKB	atgagctggtcaccttccctgacaacgcagacatgtggggcctgggaaatgaaagagcgccttgggacaggggatttggaaatgtcatccgatggcacaatcaggaaacaggt gagcagattgccatcaagcagtgccggcaggagctcagcccccggaaccgagagcggtggtgcctggagatccagatcatgagaaggctgacccaccccaatgtggtggctg cccgagatgtccctgaggggatgcagaacttggcgcccaatgacctgcccctgctggccatggagtactgccaaggaggagatctccggaagtacctgaaccagtttgagaa ctgctgtggtctgcgggaaggtgccatcctcaccttgctgagtgacattgcctctgcgcttagataccttcatgaaaacagaatcatccatcgggatctaaagccagaaaac atcgtcctgcagcaaggagaacagaggttaatacacaaaattattgacctaggatatgccaaggagctggatcagggcagtctttgcacatcattcgtggggaccctgc agtacctggccccagagctactggagcagcagaagtacacagtgaccgtcgactactggagcttcggcaccctggccttttgagtgcatcacgggcttccggcccttcctccccaactggcagcccg tgcagtggcattcaaaagtgcggcagaagagtgaggtggacattgttgttagcgaagacttgaatggaacggtgaagttttcaagctctttaccctaccccaataatcttaacagtgtcct ggctgagcgactggagaagtggctgcaactgatgctgatgtggcacccccgacagaggggcacggatcccacgtatgggcccaatggctgcttcaaggccctggatgacatcttaaactt aaagctggttcatatcttgaacatggtcacgggcaccatccacacctaccctgtgacagaggatgagagtctgcagagcttgaaggccagaatccaacaggacacgggcatcccagaggagga ccaggagctgctgcaggaagcgggcctggcgttgatccccgataagcctgccactcagtgtatttcagacggcaagttaaatgagggccacacattggacatggatcttgtttttctct ttgacaacagtaaaatcacctatgagactcagatctccccacggccccaacctgaaagtgtcagctgtatccttcaagagcccaagaggaatctcgccttcttccagctgaggaag gtgtggggccaggtctggcacagcatccagaccctgaaggaagattgcaaccggctgcagcagggacagcgagccgccatgatgaatctcctccgaaacaacagctgcctctcc aaaatgaagaattccatggcttccatgtctcagcagctcaaggccaagttggatttcttcaaaaccagcatccagattgacctggagaagtacagcgagcaaaccgagtttgggatc acatcagataaactgctgctggcctggagggaaatggagcaggctgtggagctctgtgggcgggagaacgaagtgaaactcctggtagaacggatgatggctctgcagaccgacatt gtggacttacagaggagccccatgggccggaagcagggggaacgctggacgacctagaggagcaagcaagggagctgtacaggagactaagggaaaaacctcgagaccagc gaactgagggtgacagtcaggaaatggtacggctgctgcttcaggcaattcagagcttcgagaagaaagtgcgagtgatctatacgcagctcagtaaaactgtggtttgca agcagaaggcgctggaactgttgcccaaggtggaagaggtggtgagcttaatgaatgaggatgagaagactgttgtccggctgcaggagaagcggcagaaggagctctggaatctcctgaaga ttgcttgtagcaaggtccgtggtcctgtcagtggaagcccggatagcatgaatgcctctcgacttagccagcctgggcagctgatgtctcagccctccacggcctccaacagcttacctg agccagccaagaagagtgaagaactggtggctgaagcacataacctctgcaccctgctagaaaatgccatacaggacactgtgagggaacaagaccagagtttcacggccctagact ggagctggttacagacggaagaagaagagcacagctgcctggagcaggcctcatgatgtg gggggactcgacccccctgacatggggcagcccatag caggccttgtgcagtgggggactcgacccccctgacatg gggctgcctggagcaggccgcgtgacgtggggctgcctggc

Table 2
PCR primers.

Gene name	sequence
circACTR2	F: TGGTTGGTGATGAGGCAAGT
	R: AGACCCTCCAGAAAGCACAA
miR-219a-2-3p	F: AGAATTGTGGCTGGAC
	R: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCACAGAT
IKBKB	F: GCAGGAAGTGTTTGAGGAAGTCG
	R: TGCTCACCTGTTTCCTGATTGT
ACTR2	F: CACCGGGTTTGTGAAGTGTG
	R: TGTTTCCCACTTTGGTGGTTG
GAPDH	F: TTCTTTTGCGTCGCCAGCC
	R: TCCCGTTCTCAGCCTTGACG
U6	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT

2.4. Quantitative real-time PCR (RT-qPCR)

Total RNA from BCa cells was isolated using TRIzol reagent (Invitrogen). cDNA was synthesized from extracted RNA through reverse transcription using the miScript II RT Kit (for miRNA) or M-MLV Reverse Transcriptase (for circRNAs or mRNAs) according to the manufacturer's instructions. PCR was conducted using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and the $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative RNA level with GAPDH (for circRNAs or mRNAs) or U6 (for miRNA) as internal controls. Primer sequences are listed in Table 2.

2.5. Cell counting Kit-8 (CCK-8) assay

Cells were seeded in 96-well plates at a density of 5,000 cells/well. Following the manufacturer's instructions, 10 μl of CCK-8 solution (Dojindo, Kumamoto, Japan) was mixed with the complete culture medium. This mixture then replaced the original medium at various time points (24, 48, and 72 h). After an additional 2 h of incubation, cell viability or proliferative ability was assessed with a microplate reader (Thermo Fisher Scientific, USA) by measuring optical density at a wavelength of 450 nm.

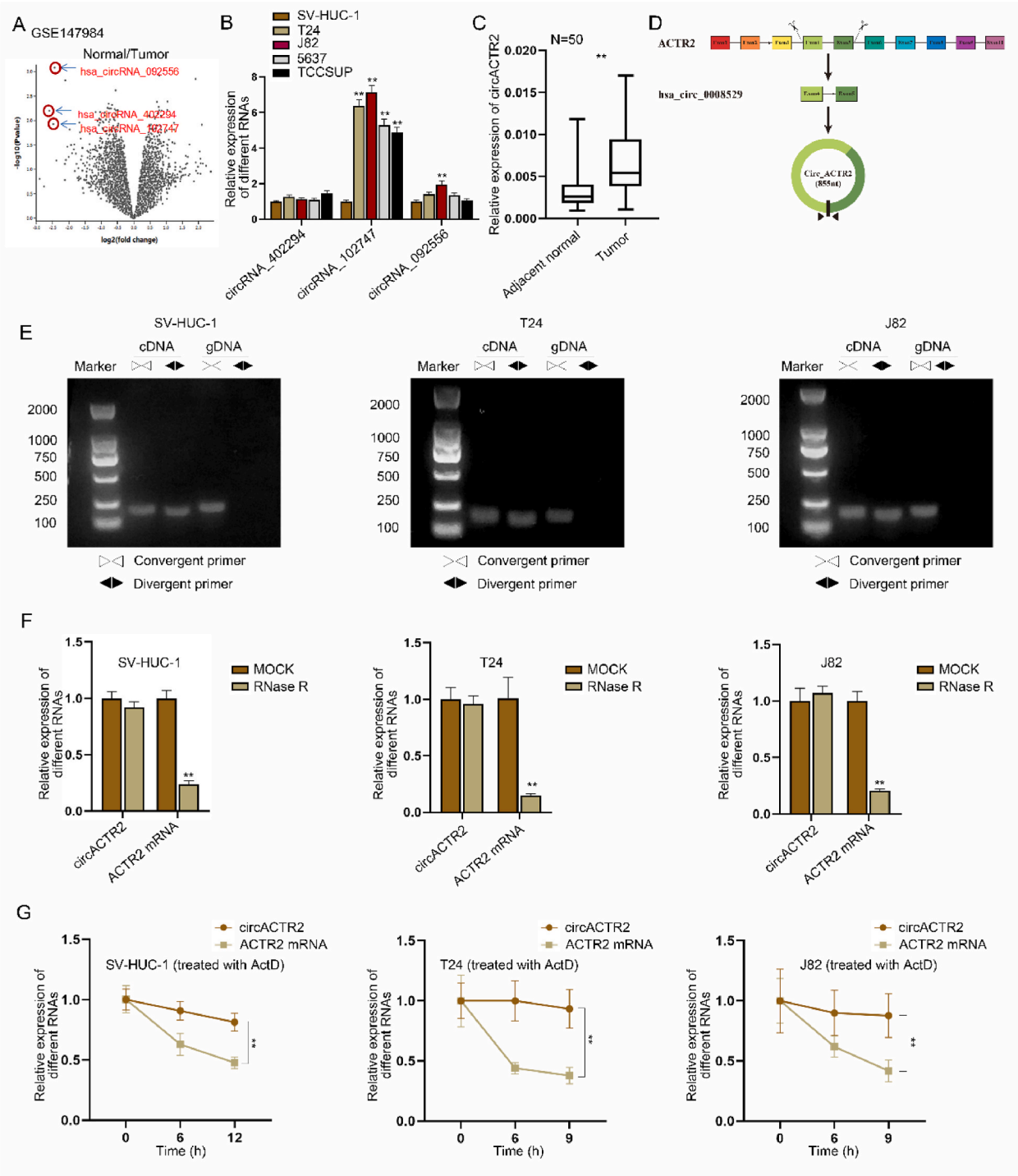

Fig. 1. CircACTR2 with circular form is upregulated in BCa tissues and cell lines. A. A volcano plot displays differentially expressed circRNAs in three pairs of tumor tissues and matched non-tumor tissues (p-value ≤0.05) according to the analysis of the GSE147984 dataset. B. The expression difference of three candidate circRNAs was detected by RT-qPCR in four BCa cell lines compared to the normal cell line (SV-HUC-1). C. The expression level of circACTR2 in 50 pairs of BCa tumor tissues and adjacent normal tissues was determined by RT-qPCR. D. The structure of hsa_circRNA_102747, derived from the ACTR2 gene, is illustrated. E. PCR and agarose gel electrophoresis were conducted to verify the amplification of circACTR2 by convergent or divergent primers in the cDNA or gDNA group. F-G. The levels of circACTR2 and linear ACTR2 mRNA were examined by RT-qPCR in SV-HUC-1 cells under RNase R or ActD treatment. **P < 0.01.

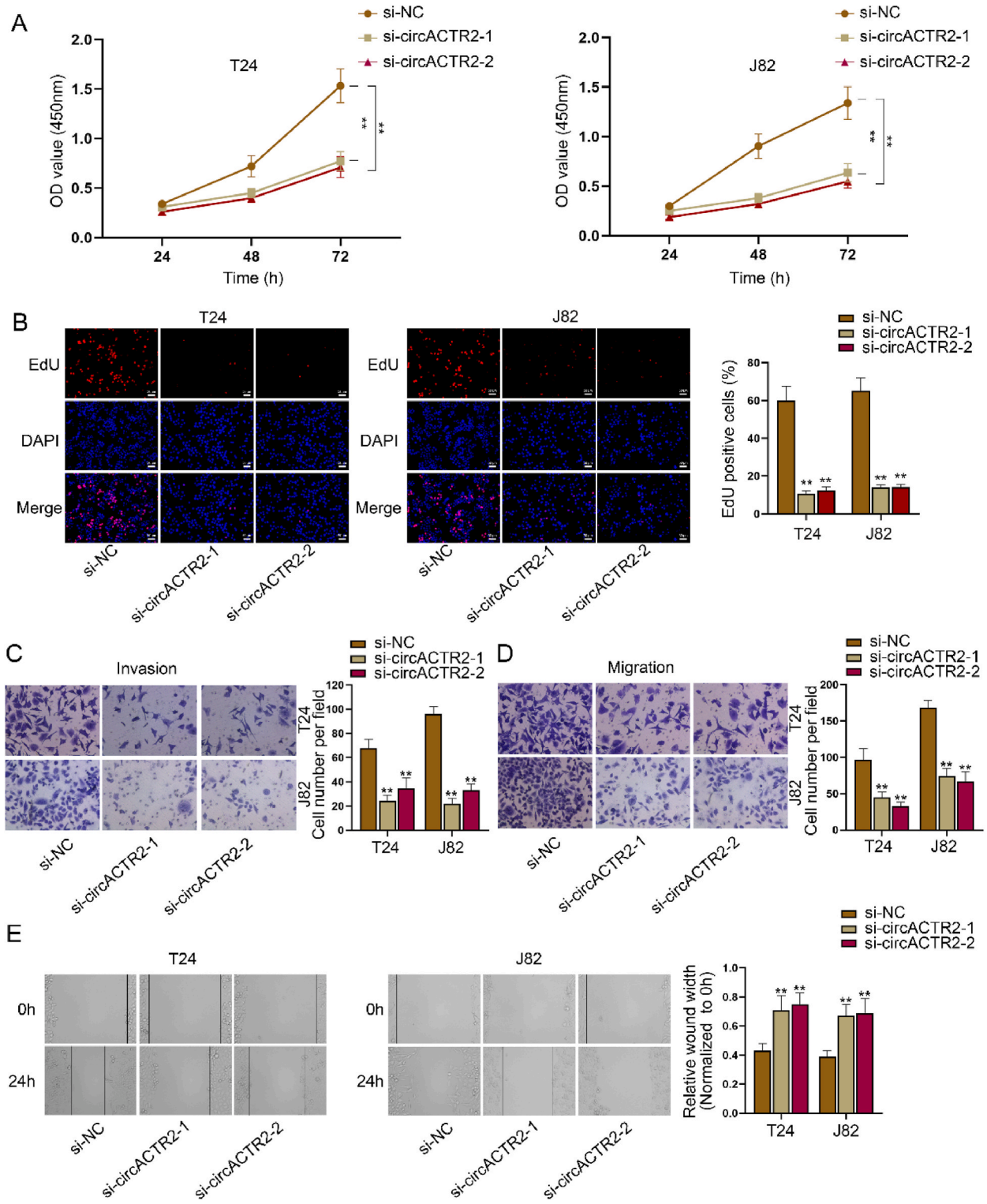

Fig. 2. CircACTR2 promotes proliferation, migration and invasion of BCa cells. T24 and J82 cells were transfected with si-NC, si-circACTR2-1, or si-circACTR2-2 for loss-of-function assays. A-B. The proliferation ability of the three groups of T24 and J82 cells was evaluated by the CCK-8 assay (A) and the EdU assay (B), respectively. C-D. The number of invaded or migrated cells was calculated using the transwell invasion (C) and migration (D) assays after being treated with the three different transfections. E. A wound healing assay was conducted on the three groups of T24 and J82 cells to assess their migration ability. **P < 0.01.

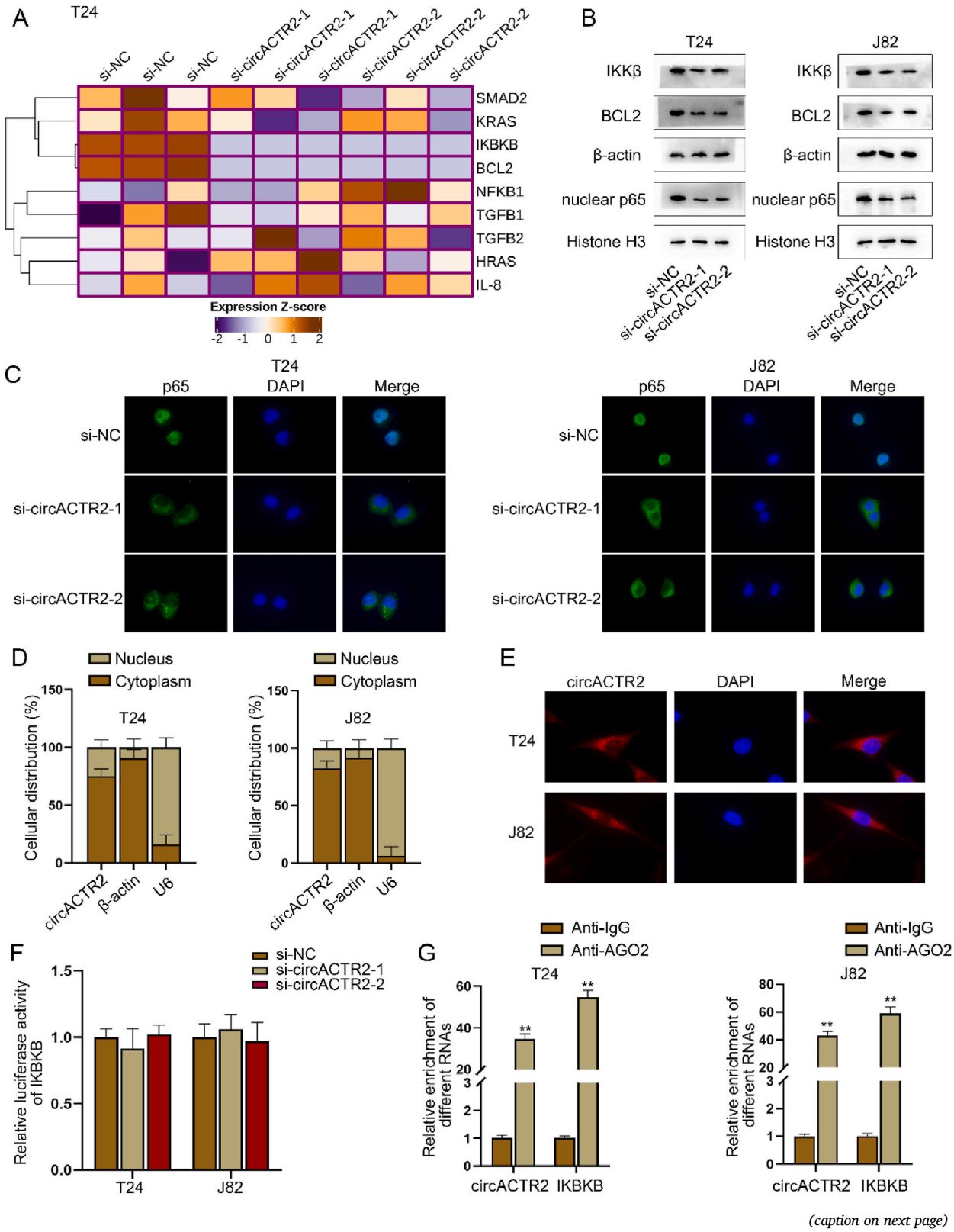

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Fig. 3. CircACTR2 modulates IKBKB-mediated NF-κB pathway. A. The expression level of key effectors of TGF-β, NF-κB, and MAPK signaling pathways was determined by RT-qPCR analysis in T24 cells with circACTR2 knockdown. B. The levels of proteins associated with the activation of the NF-κB signaling pathway were measured in circACTR2-silenced T24 and J82 cells by Western blot. C. The nuclear protein level of p65 in T24 and J82 cells transfected with si-NC or circACTR2-specific siRNAs was determined by immunofluorescence staining. D. The distribution of circACTR2 in the cytoplasm or nucleus of T24 and J82 cells was assessed by subcellular fractionation assay and analyzed by RT-qPCR. β-actin: cytoplasmic control; U6: nuclear control. E. The cellular location of circACTR2 was assessed by FISH assay. DAPI was used to stain nuclei. F. The luciferase activity of the IKBKB promoter was measured by a pGL3 luciferase reporter assay. G. The enrichment of circACTR2 and IKBKB in the immunoprecipitates of anti-AGO2 or anti-IgG (negative control) was detected by RIP assay. $^{**}P < 0.01$.

2.6. EdU assay

Transfected cells were seeded in 24-well plates at a density of 2×10^4 cells/well and cultured for 72 h. The cells were then fixed and incubated with an EdU Labeling Kit (Beyotime Biotechnology, China) for 2 h. Subsequently, cells underwent nuclear staining using DAPI, results were visualized with a fluorescence microscope (Leica, Wetzlar, Germany).

2.7. Transwell assays

Transwell assays were conducted using 24-well transwell plates (pore size: 8 μm; Corning, NY, USA) to assess migration and invasion of transfected BCa cells. For migration analysis, 4×10^4 BCa cells in serum-free medium were placed in the upper chamber, while the lower chamber contained 500 μl of 30 % FBS-containing medium. For invasion assays, chambers were pre-coated with 50 μl of Matrigel (1:6 mixture, BD Biosciences, USA). After 48 h of incubation, cells that migrated or invaded to the lower chamber were fixed and stained for observation under an inverted microscope (Olympus Corporation).

2.8. Wound healing assay

BCa cells were seeded in a six-well plate until a tight cell monolayer formed. A scratch was created in the cell monolayer using a sterile 200 μl plastic pipette tip. The cells were then washed three times with PBS to remove cell debris. After the complete medium was removed, each well was filled with serum-free medium. Twenty-four hours later, the wound width was photographed under an Olympus inverted microscope and the gap distance was calculated using Image J software.

2.9. Subcellular fractionation assay

The NUCLEI EZ PREP NUCLEI Isolation Kit (Sigma-Aldrich, St. Louis, MO, USA) was utilized to determine the cellular distribution of circACTR2 in BCa cells by isolating nuclear fractions from the cytoplasm. The isolated RNA, including circACTR2, β-actin (as a cytoplasmic control), and U6 (as a nuclear control), was reverse transcribed into cDNA for analysis by RT-qPCR.

2.10. Fluorescence in situ hybridization (FISH) assay

Briefly, T24 and J82 cells grown on slides were washed with PBS and fixed in 4 % paraformaldehyde. After treatment with a protease reagent, the slides were incubated with prehybridization buffer, then hybridized with a digoxin-labeled circACTR2 probe. Following washing, the slides were incubated with SABC-FITC at 37 °C for 30 min. The nuclei were counterstained with DAPI, and images were captured using a fluorescence microscope (Leica, Wetzlar, Germany).

2.11. Western blot

Total protein was extracted and quantified using RIPA solution and a BCA kit, respectively. The protein samples were separated on 10 % SDS-PAGE and transferred to PVDF membranes. The membranes were incubated overnight at 4 °C with primary antibodies, including IKKβ, BCL2, p65, Histone H3 (nuclear control), and β-actin (the internal control for cytoplasmic proteins). After washing thrice with TBST, the membranes were incubated with secondary antibody for 2 h. Blots were visualized using the ECL method. All antibodies were procured from Abcam (CA, USA).

2.12. RNA-binding protein immunoprecipitation (RIP)

Protein A/G agarose beads (50 μg) were incubated with 5 μg of antibodies against AGO2 or IgG (negative control) at 4 °C overnight. BCa cells were lysed, and the lysates were incubated with the antibody-bead complex at 4 °C overnight. After washing, the extracted RNA was purified for RT-qPCR analysis.

2.13. RNA pulldown assay

Biotin-labeled miR-219a-2-3p-WT containing complementary base pairs to circACTR2 or IKBKB, and miR-219a-2-3p-MUT, where the complementary base pairs were mutated, were synthesized by Ribobio (Guangzhou, China). The probes were incubated with C1

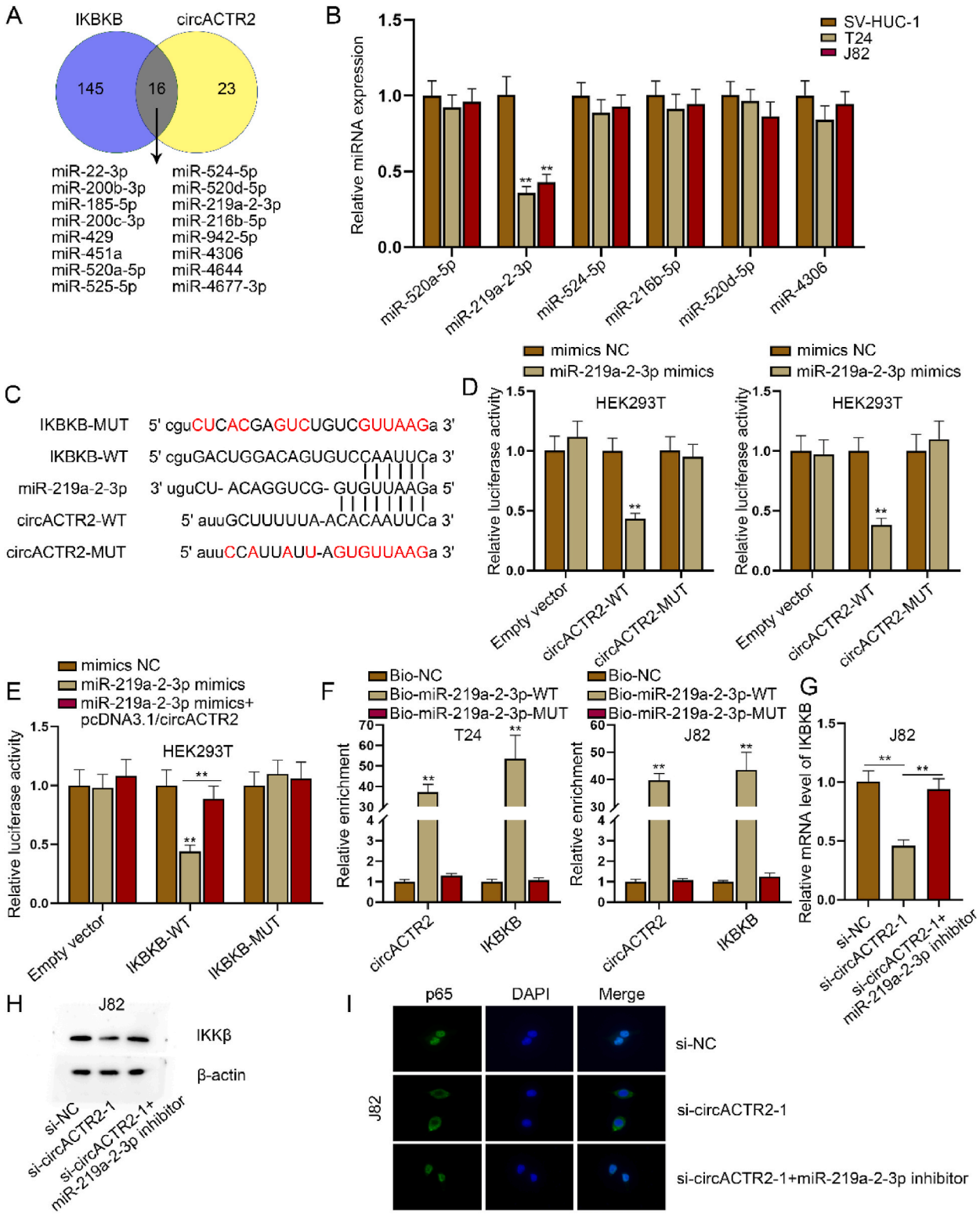

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Fig. 4. CircACTR2 increases IKBKB expression via sponging miR-219a-2-3p. A. The overlap of IKBKB-interacted miRNAs and circACTR2-interacted miRNAs is illustrated. B. Six candidate miRNAs were subjected to RT-qPCR analysis in two BCa cell lines and the normal control cell line (SV-HUC-1). C. The binding sequence of miR-219a-2-3p with circACTR2 and IKBKB; the mutant sequences of circACTR2 or IKBKB 3′UTR, which do not contain the complementary base pairs with miR-219a-2-3p, are named as circACTR2-MUT and IKBKB-MUT. D-E. A luciferase reporter assay was carried out to examine the luciferase activity of wild type reporter vectors (circACTR2-WT or IKBKB-WT) and mutant types (circACTR2-MUT or IKBKB-MUT). F. An RNA pulldown assay demonstrated the enrichment of circACTR2 and IKBKB in the pulldown products of Bio-miR-219a-2-3p-WT, Bio-miR-219a-2-3p-MUT, or Bio-NC. G-H. The levels of IKBKB mRNA and IKKβ protein, which decreased by circACTR2 knockdown, were recovered after the inhibition of miR-219a-2-3p expression, as evaluated by RT-qPCR (G) and Western blot (H). I. The reduced nuclear accumulation of p65 due to circACTR2 knockdown was also restored by silencing of miR-219a-2-3p, as detected by immunofluorescence staining. **P < 0.01.

magnetic beads (Life Technologies) at 25 °C for 2 h. Sonicated cell lysates from T24 and J82 cells were then incubated with the bead-coated probes overnight at 4 °C. After washing, RNAs were isolated with TRIzol and analyzed via RT-qPCR.

2.14. Luciferase reporter assay

The full-length sequence of circACTR2 or 3′UTR fragments of IKBKB were cloned into the pmirGLO luciferase reporter vector (Promega, Madison, WI, USA) to generate circACTR2-WT or IKBKB-WT vectors. Constructs lacking complementary base pairing with miR-219a-2-3p were used to create circACTR2-MUT or IKBKB-MUT vectors. These vectors were co-transfected into BCa cells with miR-219a-2-3p mimics or mimics-NC using Lipofectamine 2000 (Invitrogen). Forty-eight hours post-transfection, luciferase activity was measured using a Dual-Luciferase Assay System (Promega).

2.15. Statistical analysis

Data from three independent experiments were presented as mean ± SD. Differences between two groups were analyzed using Student's t-test, while one-way or two-way ANOVA was used for comparisons involving more than two groups, utilizing SPSS 22.0 software (IBM, USA). P values less than 0.05 were considered statistically significant.

3. Results

3.1. CircACTR2 with circular form is upregulated in BCa tissues and cell lines

According to the GSE147984 dataset in the GEO database (https://www.ncbi.nlm.nih.gov/gds/), three circRNAs—hsa_circRNA_102747, hsa_circRNA_092556, and hsa_circRNA_402294—are significantly upregulated in BCa tumor tissues compared to normal bladder tissues, with the threshold for significance set at $p < 0.05$ and $\log2FC < -2$ (Fig. 1A). Among these, hsa_circRNA_102747 exhibited notable upregulation in all four BCa cell lines, particularly in J82 and T24, when compared to the normal bladder epithelial cell line SV-HUC-1 (Fig. 1B), aligning with tissue sample results (Fig. 1C). Hsa_circRNA_102747, also named hsa_circ_0008529, originates from exons 4 to 5 of the ACTR2 gene and is referred to as circACTR2 in subsequent sections (Fig. 1D). To verify circACTR2's circular structure, assays were performed in the normal control cell line SV-HUC-1 and two selected BCa cell lines. Agarose gel electrophoresis of PCR products demonstrated that divergent primers could amplify circACTR2 exclusively from cDNA (Fig. 1E). Further analysis of cells treated with RNase R or ActD using RT-qPCR revealed that while the level of circACTR2 was marginally affected, ACTR2 mRNA levels significantly decreased in BCa cells following RNase R or ActD treatment (Fig. 1F and G). These findings confirm circACTR2's circular nature and its elevated presence in BCa cells.

3.2. CircACTR2 promotes proliferation, migration, and invasion of BCa cells

The biological significance of circACTR2 was investigated in BCa cells through loss-of-function assays. CircACTR2 was knocked down in two BCa cell lines, as detected by RT-qPCR (Fig. S1A). Both CCK-8 and EdU assays demonstrated that silencing circACTR2 suppressed BCa cell proliferation (Fig. 2A and B). Furthermore, the deletion of circACTR2 significantly decreased the number of migrated and invaded cells, as determined by transwell assays (Fig. 2C and D). Additionally, the wound healing assay revealed that the speed of wound closure was reduced following circACTR2 silencing (Fig. 2E), indicating an inhibition of cell migration. These findings confirm the tumor-promoting role of circACTR2 in BCa.

3.3. CircACTR2 modulates IKBKB-mediated NF-κB pathway

The molecular mechanism by which circACTR2 promotes biological functions in BCa was further explored. CircRNAs are known to mediate cancer-associated signaling pathways, such as TGF-β [11], NF-κB [12], and MAPK [13], thereby promoting BCa cell processes. To investigate the signaling pathway potentially modulated by circACTR2, we analyzed the mRNA levels of critical pathway effectors in response to circACTR2 knockdown via RT-qPCR. As shown in Fig. 3A, IKBKB and BCL2 were significantly downregulated following circACTR2 silencing. IKBKB acts as an upstream effector of the NF-κB pathway, while BCL2 is a downstream target of the NF-κB pathway. Thus, we proposed that circACTR2 modulates the NF-κB pathway through IKBKB. Knockdown of circACTR2 reduced the protein levels of IKKβ (IKBKB protein) and BCL2, as demonstrated by Western blot analysis (Fig. 3B). Additionally, the nuclear protein

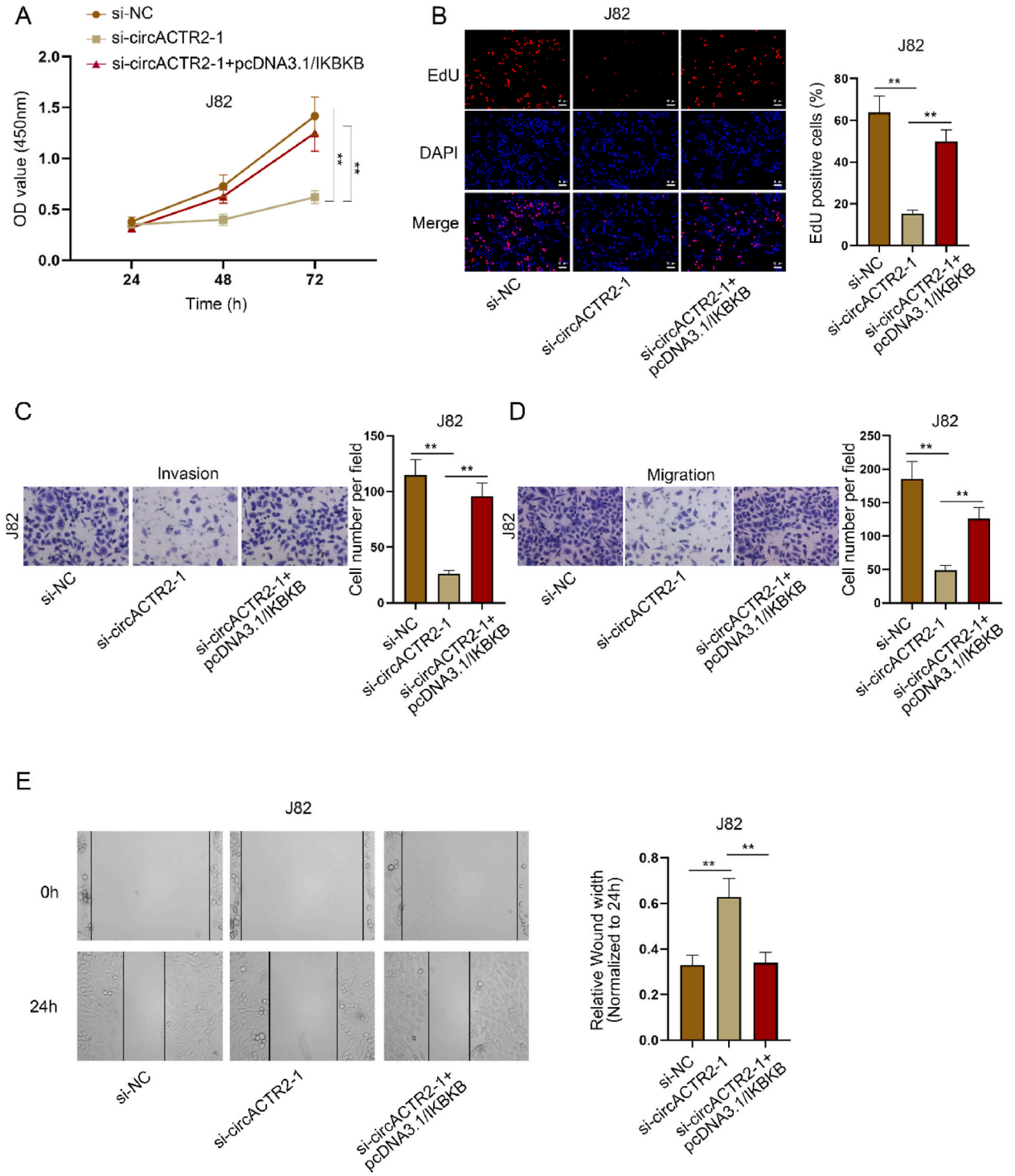

Fig. 5. IKBKB is involved in circACTR2-mediated BCa cell proliferation, invasion and migration. J82 cells were transfected with si-NC, si-circACTR2-1, or co-transfected with si-circACTR2-1 and pcDNA3.1/IKBKB for functional rescue assays. A-B. The proliferation ability of three groups of J82 cells was evaluated by CCK-8 assay (A) and EdU assay (B), respectively. C-D. The number of invaded or migrated cells was calculated using transwell invasion (C) and migration (D) assays after treatment with three different transfections. E. A wound healing assay was carried out in three groups of J82 cells to detect migration ability. **P < 0.01.

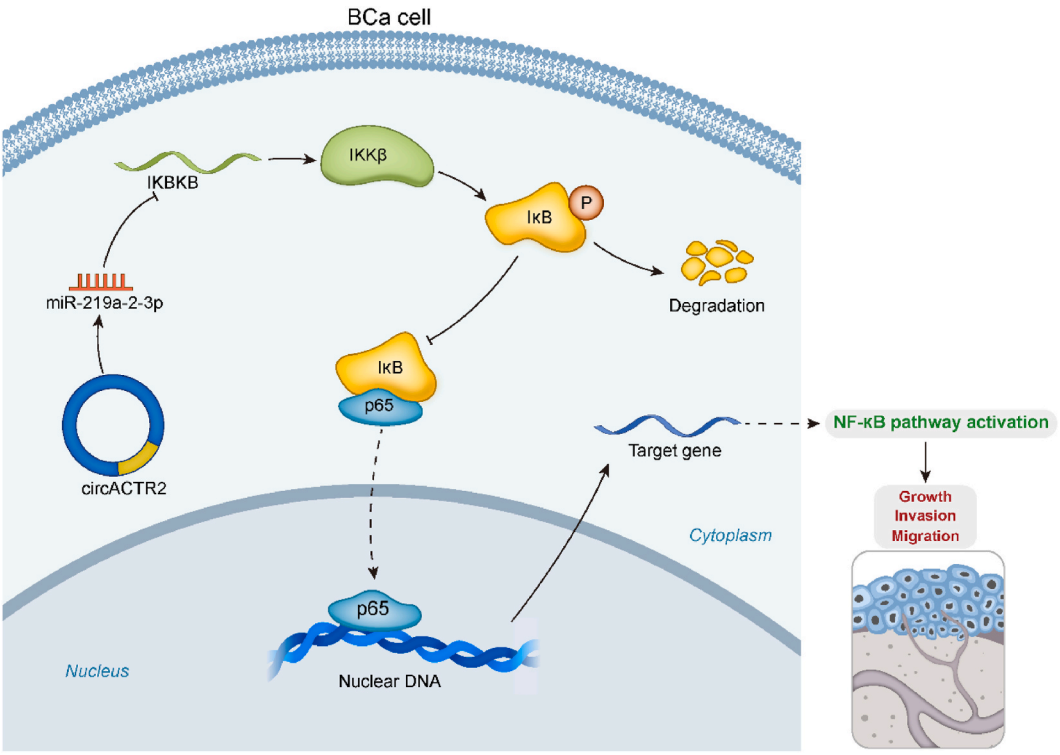

Fig. 6. A schematic map was used to illustrate that circACTR2 increases IKBKB expression and promotes BCa cell proliferation, invasion, and migration by sequestering miR-219a-2-3p to activate the NF-κB pathway.

level of p65 was also decreased by circACTR2 silencing (Fig. 3B), which was further confirmed by immunofluorescence staining (Fig. 3C). To clarify the regulatory mechanism between circACTR2 and IKBKB, we conducted subcellular fractionation and FISH assays to localize circACTR2 in BCa cells. Both assays confirmed the cytoplasmic localization of circACTR2 in BCa cells (Fig. 3D and E). We also ruled out the transcriptional regulation of IKBKB by circACTR2 through a luciferase reporter assay (Fig. 3F), suggesting a post-transcriptional regulatory role for circACTR2. The competing endogenous RNA (ceRNA) mechanism is a common post-transcriptional regulatory mode of circRNAs and mRNAs [14,15], which has been extensively reported in recent literature. Accordingly, we explored the ceRNA potential between circACTR2 and IKBKB. Moreover, both circACTR2 and IKBKB were significantly enriched in AGO2-bound immunoprecipitates (Fig. 3G), indicating their presence in the RNA-induced silencing complex (RISC). In conclusion, circACTR2 modulates IKBKB in a ceRNA manner to activate the NF-κB pathway.

3.4. CircACTR2 increases IKBKB expression via sponging miR-219a-2-3p

We sought the miRNA that could bind both circACTR2 and IKBKB. Through a search on the starBase website (https://starbase.sysu.edu.cn/), we identified 16 candidate miRNAs (Fig. 4A). Among these candidates, miR-520a-5p, miR-524-5p, miR-520d-5p, miR-219a-2-3p, miR-216b-5p, miR-942-5p, and miR-4306 have been reported as tumor suppressors in various types of human cancers. MiR-942-5p was excluded because it has been shown to target IKBKB directly. The remaining six miRNAs were analyzed by RT-qPCR in two BCa cell lines and one normal control cell line. Ultimately, miR-219a-2-3p was selected for further investigation as it was downregulated in both BCa cell lines (Fig. 4B). To confirm its interaction with circACTR2 and IKBKB, we mapped its binding sequences on the IKBKB 3′UTR and circACTR2 and mutated these sequences on circACTR2 or IKBKB 3′UTR to disrupt their interactions with miR-219a-2-3p (Fig. 4C). The results showed that the luciferase activity of reporter vectors containing the wild-type sequences of circACTR2 or IKBKB 3′UTR (circACTR2-WT or IKBKB-WT) was reduced upon co-transfection with the miR-219a-2-3p overexpression vector, whereas the activity of reporter vectors containing mutant sequences (circACTR2-MUT or IKBKB-MUT) remained unchanged (Fig. 4D and E). RNA pulldown assays further indicated that circACTR2 and IKBKB were enriched in the pulldown products of Bio-miR-219a-2-3p-WT compared to Bio-miR-219a-2-3p-MUT or Bio-NC (Fig. 4F). Inhibition of miR-219a-2-3p expression could counteract the decrease in IKBKB mRNA and IKKβ protein levels mediated by circACTR2 knockdown (Fig. 4G and H). Similarly, the reduction in nuclear p65 accumulation induced by circACTR2 knockdown was reversed by silencing miR-219a-2-3p (Fig. 4I). In conclusion, circACTR2 upregulates IKBKB expression and promotes p65 nuclear translocation by sponging miR-219a-2-3p.

3.5. IKBKB is involved in circACTR2-mediated BCa cell proliferation, invasion, and migration

To demonstrate that circACTR2's functions in BCa cells depend on IKBKB, we overexpressed IKBKB in J82 cells for rescue assays (Fig. S1B). Functionally, the suppression of cell proliferation by circACTR2 silencing was reversed by IKBKB overexpression (Fig. 5A and B). Moreover, the recovery in cell invasion and migration, previously inhibited by circACTR2 knockdown, was facilitated by IKBKB overexpression (Fig. 5C–E). To summarize, circACTR2 promotes cell proliferation, invasion, and migration in BCa in an IKBKB-dependent manner.

4. Discussion

BCa is a heterogeneous disease characterized by diverse clinical behaviors and carcinogenic mechanisms [16]. Recently, numerous circRNAs have been identified as diagnostic or prognostic biomarkers and even potential therapeutic targets in BC [17]. Previous studies have classified circRNAs as either oncogenic factors or tumor suppressors in BCa [18,19]. In our research, we identified a circRNA potentially associated with BCa progression using the GEO database (GSE147984 dataset). Consistent with GEO dataset analysis, circACTR2 was found to be significantly overexpressed in BCa cells. To further validate this, we assessed circACTR2 expression in clinical samples from 50 BCa patients diagnosed at our hospital, revealing circACTR2 upregulation in tumor tissues, which suggests its clinical significance in BCa. The circular structure of circACTR2 was confirmed through its resistance to RNase R digestion and ActD treatment, along with specific amplification in cDNA using divergent primers, thus establishing its circular nature and potential role in BCa progression. Although previous research has shown circACTR2's involvement in renal fibrosis and diabetic nephropathy [8–10], its function in BCa cell processes remains unexplored. Our study is the first to demonstrate that circACTR2 enhances BCa cell proliferation, migration, and invasion.

Classical signaling pathways like MAPK, Wnt, PI3K/AKT, TGF-β, and NF-κB are crucial modulators in tumor progression [20–24]. We revealed that circACTR2 modulate NF-κB signaling pathway effectors in BCa cells, Among IKBKB (IKKβ), which mediates IκBα phosphorylation, releasing p65 and thus activating the NF-κB signaling pathway [25]. In this study, we found that circACTR2 could upregulate IKBKB in BCa cells and induce nuclear accumulation of p65 to activate the NF-κB signaling pathway.

CircRNAs are recognized as ceRNAs that sponge miRNAs to upregulate downstream mRNAs, thereby facilitating tumor progression [26,27]. Our findings indicate circACTR2's predominant cytoplasmic distribution and enrichment in the RISC along with IKBKB, excluding its transcriptional regulation on IKBKB. This suggests circACTR2's ceRNA activity to upregulate IKBKB in BCa cells. Furthermore, miR-219a-2-3p was predicted to bind both circACTR2 and IKBKB. Previous studies identified miR-219a-2-3p as a tumor suppressor and a circRNA sponge [28–31]. Yet its role in BCa was unclear. Our study is the first to elucidate its interaction with circACTR2 and IKBKB in a ceRNA manner, and through rescue assays, we showed that circACTR2's effects in BCa cells rely on IKBKB.

In summary, our study highlights the oncogenic role of circACTR2 in BCa by sponging miR-219a-2-3p to upregulate IKBKB and activate the NF-κB signaling pathway (Fig. 6), offering a new target for BCa treatment. Future work will aim to uncover additional mechanisms by which circACTR2 influences BCa progression.

Ethical statement

Clinical study was approved by the Ethical Committee of Jinling Hospital, Jinling School of Clinical Medicine, Nanjing Medical University (20200603114).

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CRediT authorship contribution statement

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30778.

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