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Circ_0002623 promotes bladder cancer progression by regulating the miR-1276/SMAD2 axis

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Abstract

Circular RNAs (circRNAs) are key regulatory factors in the development of multiple cancers. This study is targeted at exploring the effect of circ_0002623 on bladder cancer (BCa) progression and its mechanism. Circ_0002623 was screened out by analyzing the expression profile of circRNAs in BCa tissues. Circ_0002623, miR-1276, and SMAD2 mRNA expression levels in clinical sample tissues and cell lines were detected through quantitative real-time polymerase chain reaction (qRT-PCR). After circ_0002623 had been overexpressed or silenced in BCa cells, the cell proliferation, migration, and cell cycle were evaluated by CCK-8, BrdU, Transwell assay, and flow cytometry. Tumor xenograft model was used to validate the biological function of circ_0002623 in vivo. Bioinformatics analysis and dual-luciferase reporter gene assay were conducted for analyzing and confirming, respectively, the targeted relationship between circ_0002623 and miR-1276, as well as between miR-1276 and SMAD2. The regulatory effects of circ_0002623 and miR-1276 on the expression levels of TGF-β, WNT1, and SMAD2 in BCa cells were detected by Western blot. We reported that, in BCa tissues and cell lines, circ_0002623 was upregulated, whereas miR-1276 was downregulated. Circ_0002623 positively regulated BCa cell proliferation, migration, and cell cycle progression. Additionally, circ_0002623 could competitively bind with miR-1276 to increase the expression of SMAD2, the target gene of miR-1276. Furthermore, circ_0002623 could regulate the expression of TGF-β and WNT1 via modulating miR-1276 and SMAD2. This study helps to better understand the molecular mechanism underlying BCa progression.

KEYWORDS
BCa, Circ_0002623, MiR-1276, SMAD2

Abbreviations: ATCC, American Type Culture Collection; BCa, bladder cancer; CCK-8, Cell Counting Kit-8; cDNA, complementary DNA; circRNA, circular RNA; DMSO, dimethylsulphoxide; EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum; GEO, Gene Expression Omnibus; MiRNA, microRNA; PBS, phosphate buffer saline; qRT-PCR, quantitative real-time polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SMAD2, SMAD family member 2; VANGL1, VANGL planar cell polarity protein 1; WHO, World Health Organization.

Zhaocun Zhang and Haifeng Zhao are co–first authors.

1 | INTRODUCTION

Known as the second most common malignancy of the urinary system, bladder cancer (BCa) causes around 429 800 new cases and 165 100 deaths globally every year.[1,2] The current treatment strategy for BCa is surgical removal combined with adjuvant chemotherapy.[3,4] Unfortunately, nearly 50% of patients undergo recurrence/metastases within 2 years after receiving surgery.[5] In this context, it is crucial to dig into the molecular mechanisms related to BCa progression and metastasis.

Recognized as a type of single-stranded noncoding RNA with a covalently closed-loop structure, circular RNAs (circRNAs) are formed by reverse splicing of precursor messenger RNA (pre-mRNA) and are without the 5′-end cap structure and 3′-end polyadenylic acid tail shared by linear RNAs. circRNA is generated by the circularization of one or several exons in the sequence of pre-mRNA: The end of an exon binds to the beginning of an upstream exon.[6] The closed-loop structure prevents them from being degraded by exoribonuclease (RNase R).[6] Due to their specific expression patterns and biological functions in tumors, circRNAs have the potential to be tumor biomarkers and treatment targets.[7,8] There have been some studies reporting the relationship between circRNAs and BCa progression. For instance, circ-ZKSCAN1 is significantly upregulated in BCa, which is strongly associated with disease progression.[7]

MicroRNAs (miRNAs or miRs) inhibit messenger RNA (mRNA) translation or promotes mRNA degradation, regulating the progression of multiple cancers.[9] There is growing evidence that miRNAs participate in regulating BCa progression. For example, miR-433-3p modulates BCa cell growth, migration, and invasion by repressing CCAR1 expression.[10] It is reported that miR-1276 is downregulated in gastric carcinoma and exerts a tumor-inhibiting effect,[11] yet the role and mechanism of miR-1276 in BCa are unclear.

We analyzed the GEO dataset GSE92675 to screen out the differentially expressed circRNAs in BCa. It was observed that circ_0002623 expression was significantly enhanced in BCa in comparison with adjacent normal tissues. Circ_0002623 is generated from VANGL planar cell polarity protein 1 (VANGL1) transcript, and interestingly, accumulating studies support that VANGL1 and VANGL1-derived circRNA are cancer promoters.[12,13] In the present work, we investigated the expression characteristics, clinical significance, biological function, and underlying mechanism of circ_0002623 in BCa. It is found that circ_0002623, which is highly expressed in BCa tissues and cells, promotes the malignant phenotypes of BCa cells and regulates the expression levels of miR-1276 and SMAD family member 2 (SMAD2).

2 | MATERIALS AND METHODS

2.1 | Patient specimens

Thirty-two pairs of BCa tissues and paracancerous tissues of patients were collected from Qilu Hospital of Shandong University and immediately stored in liquid nitrogen. None of the patients received chemotherapy or radiotherapy before tumorectomy. The BCa grade was determined according to WHO's histological tumor-grading criteria, and the BCa stage was evaluated in accordance with the TNM classification of malignancies by the Union for International Cancer Control. The current study was endorsed by the ethics committee of Qilu Hospital of Shandong University, and each patient signed a written informed consent.

2.2 | Analysis of gene expression profile

From the GEO database (http://www.ncbi.nlm.nih.gov/geo/), with the keywords ("circRNA" and "bladder cancer"), the dataset GSE92675 was obtained.[12] The GEO2R online analysis tool was then used to analyze the data. Circular RNAs with $p < .05$ and $|\log2(\text{Fold Change})| > 2$ were considered the differentially expressed circRNAs.

2.3 | Cell culture and transfection

Human BCa cell lines (J82, RT4, T24, 5637, and HT-1376) and immortalized bladder epithelial cells (SV-HUC-1) were bought from the American Type Culture Collection (ATCC) and the Chinese Academy of Sciences. All cells were cultured in RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (FBS; Gibco), 0.1 mg/mL streptomycin, and 100 U/mL penicillin (Invitrogen) in an incubator containing 5% CO_2 at 37°C. Circ_0002623 siRNA (si-circ_0002623#1: 5′-GACACCTGAGGGAAAGAACTA-3′ and si-circ_0002623#2: 5′-GGACACCTGAGGGAAAGAACT-3′, designed according to CircInteractome database), scramble siRNA (si-NC), miR-1276 mimics/miR-1276 inhibitors, and the negative control (mimics NC/inhibitors NC) were purchased from Invitrogen (Carlsbad). The siRNAs targeting SMAD2 were bought from Santa Cruz Biotechnology. The BCa cells were transfected with Lipofectamine® 2000 (Invitrogen) according to the manufacturer's instructions. Lentiviruses system (GeneChem) was used to establish T24 cells which stably overexpressed circ_0002623. Circ_0002623 overexpression plasmids (pLV-circ-puro VL3531, Figure S1) were designed and provided by Inovogen Tech. The sequences of VANGL1 exon 3 and exon 4 were inserted into the empty vector. This vector could also express two sequences with complementary pairing relationship, which promoted the circularization of the transcript.

2.4 | Quantitative RT-PCR

The total RNA of tissues or cells was extracted with TRIzol reagent (Thermo Fisher Scientific). The TaqMan MicroRNA reverse transcription kit (Applied Biosystems) was used to perform the cDNA synthesis for miR-1276, and the cDNA synthesis for SMAD2 and circ_0002623 was conducted with the PrimeScript RT Master Mix Kit (Takara Biotechnology Co., Ltd.). Subsequently, with the SYBR® Premix Ex Taq™ II kit (Takara Biotechnology Co., Ltd.) and with cDNA as the template, the relative expressions of SMAD2 and

circ_0002623 were determined by qRT-PCR. In addition, the stem-loop primer SYBR Green qRT-PCR kit (Synbio Tech) was used for qRT-PCR to evaluate miR-1276 expression. With U6 and GAPDH as internal references, the relative expressions of circ_0002623, miR-1276, and SMAD2 were calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed in Table 1.

2.5 | Actinomycin D and RNase R treatment

The cell culture medium was added with 2 mg/mL Actinomycin D or dimethylsulphoxide (DMSO) (Sigma-Aldrich) as a negative control to block transcription of BCa cells. Total RNA (2 μg) was incubated for 30 minutes at 37°C with or without 3 U/μg RNase R (Epicentre Technologies). Following the Actinomycin D and RNase R treatment, circ_0002623 and GAPDH expression levels were determined by qRT-PCR, respectively.

2.6 | Cell Counting Kit-8 (CCK-8) assay

J82 and 5637 cells were harvested, and resuspended, and the cell density was adjusted to 1×10^4 cells/mL. Next, the cells were transferred into 96-well plates (100 μL of cell suspension/well), and the cells were cultured. At 24, 48, 72, and 96 hours, each well was added with 10 μL of CCK-8 solution (Beyotime Biotechnology). After 1 hour of incubation, a microplate reader was utilized for determining the absorbance of each well at 450 nm wavelength.

2.7 | BrdU proliferation assay

Cells were transferred at 1×10^5 cells/mL into the 35-mm-diameter petri dish with a glass coverslip in it, cultured for 1 day, and synchronized with medium containing 0.4% FBS for 3 days so as to enable the majority of cells to be in G_0 phase. Then, the cells were cultured at 37°C for 2 hours with complete medium and 1.0 mg/mL BrdU reagent (BD Pharmingen). After that, the medium was discarded, and the slides were washed three times in phosphate buffer saline (PBS), followed by the fixation of the cells with methanol for 10 minutes. Then 5% normal rabbit serum was used to block the cells, and formamide was used to denature nucleic acids. Subsequently, the cells were incubated at 37°C with primary antibody (1:500, cat no. ab152095, Abcam) for 1 hour at room temperature, while the control group was incubated with PBS. Next, the nuclei of the cells were stained with DAPI staining solution. Finally, the cells in 10 visual fields were observed and counted under a fluorescence microscope (magnification, ×200; Leica Microsystems GmbH).

TABLE 1 Primer sequences

Name	Primer sequence
circ_0002623	Forward: 5′-CGAGTCCCGCTTCTACAGC-3′
	Reverse: 5′-CTCTCCAGTGGGAGGTTGAA-3′
miR-1276	Forward: 5′-TAGGTAAAGAGCCCTGTGGAGA-3′
	Reverse: 5′-CATCAAGGCCCAAGTGCTCAG-3′
SMAD2	Forward: 5′-ACTAACTTCCCAGCAGGAAT-3′
	Reverse: 5′-GTTGGTCACTTGTTTCTCCA-3′
U6	Forward: 5′-CTCGCTTCGGCAGCACATATACT-3′
	Reverse: 5′-ACGCTTCACGAATTT-GCGTGTC-3′
GAPDH	Forward: 5′-CATGAGAAGTATGACAACAGCCT-3′
	Reverse: 5′-AGTCCTTCCACGATACCAAAGT-3′

2.8 | Transwell assay

Transwell chamber (Corning Life Sciences) was employed for detecting cell migration capability. Transfected BCa cells during logarithmic growth stage were harvested. The single-cell suspension was prepared with serum-free medium, and the cell concentration was adjusted to 1×10^5 cells/mL. A total of 200 μL of cell suspension was added to the upper chamber, and 500 μL of medium containing 10% FBS was added to the lower chamber. The cells were then cultured for 24 hours. Subsequently, the cells remaining on the upper surface of the filter were removed, and the cells in the below surface of the filter were fixed with methanol and stained with 0.1% crystal violet solution. Finally, five visual fields were randomly selected under the microscope, and the BCa cells which had passed through the membrane were counted.

2.9 | Cell cycle analysis

Briefly, in each sample, 1×10^6 cells were fixed with 75% ice-cold ethanol and then treated for 30 minutes with 2 μg/mL RNase (Sigma-Aldrich). Next, 20 mg/mL propidium iodide (BD Biosciences) was used for staining these cells. After the cells had been washed by PBS, a FACSCalibur flow cytometry (BD Biosciences) was utilized to analyze cell cycle distribution. ModFit LT v2.0 software was used for data analysis.

2.10 | Western blot assay

Bladder cancer cells were lysed for 20 minutes with 1 mL of RIPA lysis buffer (Biosharp Life Sciences) on ice. The obtained mixture was then centrifuged at 11000*g* at 4°C for 10 minutes, and the supernatant was collected. The BCA assay kit (Thermo Fisher Scientific) was adopted to measure protein concentration in the supernatant. Subsequently, the cell lysates were mixed with loading buffer and denatured in boiling water. Next, the proteins were dissolved in SDS-PAGE and then transferred onto PVDF membranes. After the membranes had been blocked with 5% skim milk at room temperature for 30 minutes, they were incubated overnight with anti-WNT1 antibody (1:1000; ab228526; Abcam), anti-SMAD2 antibody

(1:1000; ab40855; Abcam), anti-GAPDH antibody (1:1000; ab9485; Abcam), and anti-TGF-β antibody (1:1000; ab179695; Abcam) at 4°C overnight. Next, the membranes and the horseradish peroxidase–conjugated secondary antibody (1:2000; ab205718; Abcam) were incubated for 1 hour at room temperature. Eventually, ECL Western blotting substrate (Promega) was used to develop the protein bands.

2.11 | Luciferase reporter assay

The binding fragments of circ_0002623 and SMAD2 3'UTR with miR-1276 were amplified by PCR. The amplification products were inserted into the PGL3-promoter vector (Promega,) to construct circ_0002623 and SMAD2 wild-type (wt) luciferase reporter plasmids; accordingly, with site-directed mutation, circ_0002623 and SMAD2 mutant type (mut) luciferase reporter plasmids were constructed. The recombinant plasmids were cotransfected with miR-1276 and mimics negative control (NC) into J82 and 5636 cells, respectively. After 48 hours of transfection, the cells were collected. Following the manufacturer's instruction, a dual-luciferase reporter gene assay system (Promega) was used to detect the luciferase activity.

2.12 | Tumor xenograft assay and in vivo metastasis assay

The Ethics Committee of Qilu Hospital approved all animal experiments. From the Slac Laboratory Animals Ltd., 4-week-old male BALB/c nude mice were bought. The right flank of the mice (n = 3 in each group) was subcutaneously injected with 1×10^7 T24 cells (with circ_0002623 overexpression or not), and every week the tumor size was measured. The mice were euthanized after four weeks, and the tumors were removed and weighed. For the tumor metastasis assay in vivo, the nude mice (n = 10 in each group) were injected through the caudal vein with the T24 cells. All the mice were euthanized after 4 weeks, and their lungs were surgically removed. The samples were embedded in paraffin for hematoxylin and eosin (HE) staining. The severity of lung metastasis was evaluated by pathologists.

2.13 | Statistical analysis

All of the experiments were performed in triplicate, the data were expressed as mean ± standard deviation, and statistical analysis was performed with GraphPad Prism 8 (GraphPad Software, Inc.). One-way ANOVA was utilized for the comparison of the differences among multiple groups, and *t* test was conducted to make the comparisons between two groups. The correlation between the patients' pathological factors and circ_0002623 expression was analyzed using Fisher's exact test. Pearson's correlation coefficient was applied for the correlation analysis. The survival analysis was performed by the Kaplan-Meier method and analyzed by log-rank test. When $p < .05$, a difference was of statistical significance.

3 | RESULTS

3.1 | Circ_0002623 is highly expressed in BCa

First, the circRNA expression data in GSE92675 were analyzed. The volcano plot showed the expression patterns of circRNAs in the dataset, of which 26 are downregulated circRNAs and 84 are upregulated circRNAs (Figure 1A). The heat map displayed the significantly upregulated circRNAs ($p < .05$ and log2[Fold Change] > 2) (Figure 1B). Circ_0002623 was one of the upregulated circRNAs in BCa tissues with the most significant fold change (Figure 1A-C). Bioinformatics analysis and Sanger sequencing showed that circ_0002623 was derived from exons 3-4 of the VANGL1 transcript by back-splicing (Figure 1D). Next, qRT-PCR was conducted for detecting circ_0002623 expression in paired BCa tissues and adjacent tissues of 32 patients, and it was revealed that circ_0002623 expression in BCa tissues was significantly higher as opposed to paracancerous tissues (Figure 1E). We further analyzed the relationship between circ_0002623 expression and BCa patients' pathological features, and it was revealed that high circ_0002623 expression was positively correlated with lymph node metastasis of the patients (Table 2). Additionally, high circ_0002623 expression was significantly associated with a shorter overall survival of BCa patients (Figure 1G). In contrast to immortalized human bladder epithelial cells (SV-HUC-1), circ_0002623 expression in BCa cell lines (RT4, T24, J82, 5637, and HT-1376) was significantly elevated (Figure 1F).

3.2 | Confirmation of the circular structure and subcellular localization of circ_0002623

The subcellular localization and stability of circ_0002623 in 5637 and J82 cells were then investigated. After treatment with Actinomycin D, an inhibitor of transcription, total RNA from 5637 and J82 cells was isolated at the indicated time points. Subsequently, circ_0002623 and GAPDH expression levels were measured by qRT-PCR, and it was revealed that the half-life of circ_0002623 was longer than 24 hours, while that of GAPDH was approximately 4 hours in both 5637 and J82 cells (Figure 2A). Additionally, circ_0002623 was resistant to the degradation mediated by RNase R (Figure 2B). These data supported that circ_0002623 was a circular RNA. Next, the RNA from cytoplasmic and nuclear fractions of BCa cells was respectively extracted, and qRT-PCR showed that circ_0002623 was predominantly located in the cytoplasm of 5637 and J82 cells (Figure 2C).

3.3 | Circ_0002623 knockdown inhibits BCa cell proliferation, migration, and cell cycle

To construct circ_0002623 knockdown models for the follow-up experiments, 5637 and J82 cell lines were selected (Figure 3A). It was revealed that circ_0002623 knockdown significantly restrained

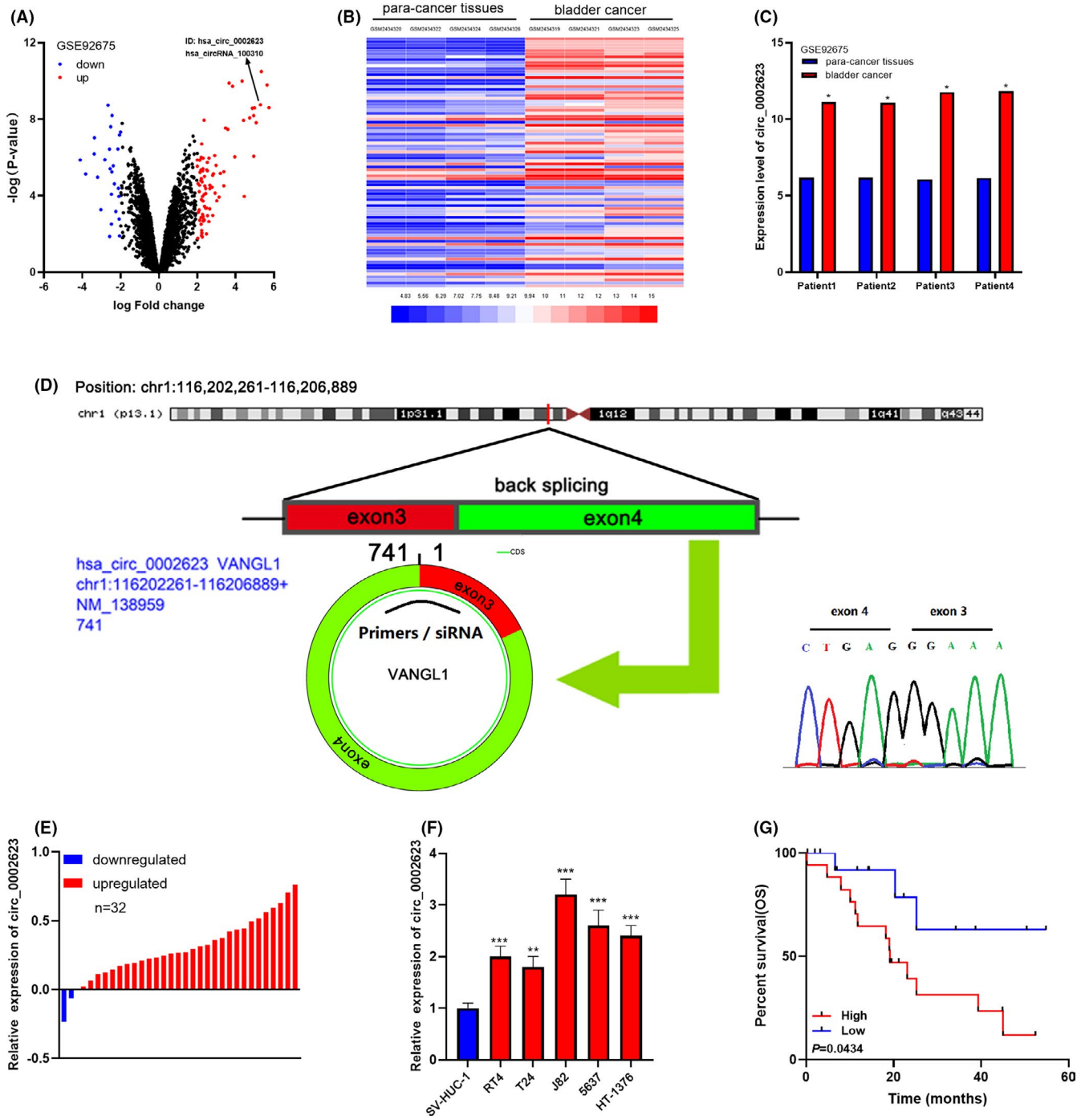

FIGURE 1 Circ_0002623 is upregulated in bladder cancer (BCa). A, Volcano plot was used to analyze and screen out circular RNAs (circRNAs) that are significantly upregulated and downregulated in BCa. Red represents upregulated circRNAs, and blue represents downregulated circRNAs. B, A heat map was used to show the expression profiles of circRNAs which were significantly upregulated in BCa. C, Circ_0002623 expression in four pairs of adjacent tissues and BCa tissues in the GSE92675 dataset. D, Bioinformatics analysis and Sanger sequencing showed that circ_0002623 was formed by reverse splicing of the exons of the VANGL1 transcript. E, Quantitative RT-PCR was used to detect circ_0002623 expression in BCa tissues and paracancerous tissues. F, Quantitative RT-PCR was used to detect circ_0002623 expression in immortalized bladder epithelial cells (SV-HUC-1) and BCa cell lines (RT4, T24, J82, 5637, and HT-1376). G, Kaplan-Meier curve was used to analyze the relationship between circ_0002623 expression and BCa patients' overall survival. $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$

J82 and 5637 cell viability and proliferation (Figure 3B,C). Knocking down circ_0002623 also blocked the migration of J82 and 5637 cells (Figure 4A). Flow cytometry suggested that knocking down circ_0002623 impeded cell cycle progression of BCa cells, giving rise to more cells being blocked at G0/G1 stage and thus fewer cells in S and G2/M stages (Figure 4B). Consistently, circ_0002623 overexpression in T24 cells facilitated proliferation, migration, and cell cycle progression (Figure S2A-E).

TABLE 2 The relationship between circ_0002623 and clinicopathological indicators in patients with bladder cancer

Expression of circ_0002623				
Pathological factors	Low (16)	High (16)	Chi-square value	*p* value
Gender			0.5818	.704
Male	10	12		
Female	6	4		
Age			0.5333	.716
<55	11	9		
>55	5	7		
Lymph node metastasis			6.1490	.032*
Absent	11	4		
Present	5	12		
Grade			4.8000	.066
Low	9	3		
High	7	13		
T stage			4.5714	.073
T1-T2	12	6		
T3-T4	4	10		

Note: Fisher's exact test.

*$p < .05$

3.4 | Circ_0002623 promotes BCa progression by targeting miR-1276

There is growing evidence showing that circRNAs, as competitive endogenous RNA (ceRNA), can bind to the miRNA to regulate its functions and play a role in tumors.[14,15] StarBase (http://starbase.sysu.edu.cn/index.php) and Circular RNA Interactome (https://circinteractome.nia.nih.gov/) were searched for predicting candidate miRNAs with complementary sequences to circ_0002623, and a total of eight miRNAs were found; miR-1276 (context++ score = 94) was selected for the follow-up research (Figure 5A,B). The binding site of circ_0002623 and miR-1276 was obtained from the above databases (Figure 5C). Dual-luciferase reporter gene assay showed that miR-1276 overexpression could reduce the luciferase activity of the cells cotransfected with circ_0002623-wt but had no significant effect on the luciferase activity of the cells cotransfected with circ_0002623-mut (Figure 5D). Quantitative RT-PCR indicated that knocking down circ_0002623 in J82 and 5637 cells gave rise to a significant increase in miR-1276 expression (Figure 5E). Moreover, miR-1276 expression in BCa tissues and cell lines was remarkably decreased (Figure 5F,G). Additionally, in BCa tissues,

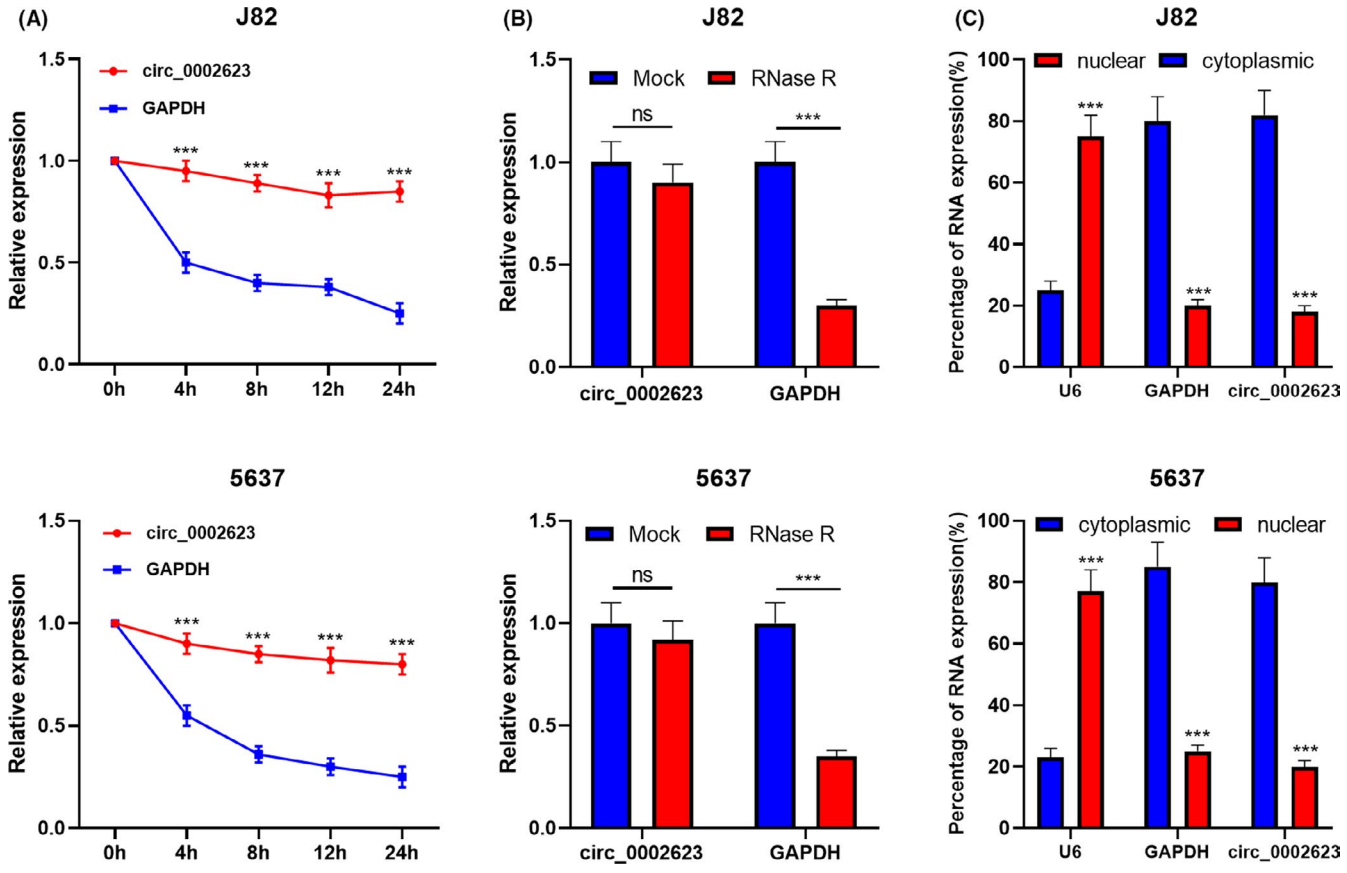

FIGURE 2 Characteristics of circ_0002623 in bladder cancer (BCa) cells. A, Quantitative RT-PCR was performed to detect circ_0002623 and GAPDH expression in J82 and 5637 cells after the cells had been treated with Actinomyci D at the indicated time points. B, Quantitative RT-PCRwas performed to detect circ_0002623 and GAPDH expressions in J82 and 5637 cells after the total RNA had been treated with or without RNase R. C, Quantitative RT-PCR was performed to detect circ_0002623 expression in the nuclear and cytoplasmic fractions. U6 snRNA and GAPDH acted as positive controls for nuclear and cytoplasmic fractions, respectively. ***$p < .001$

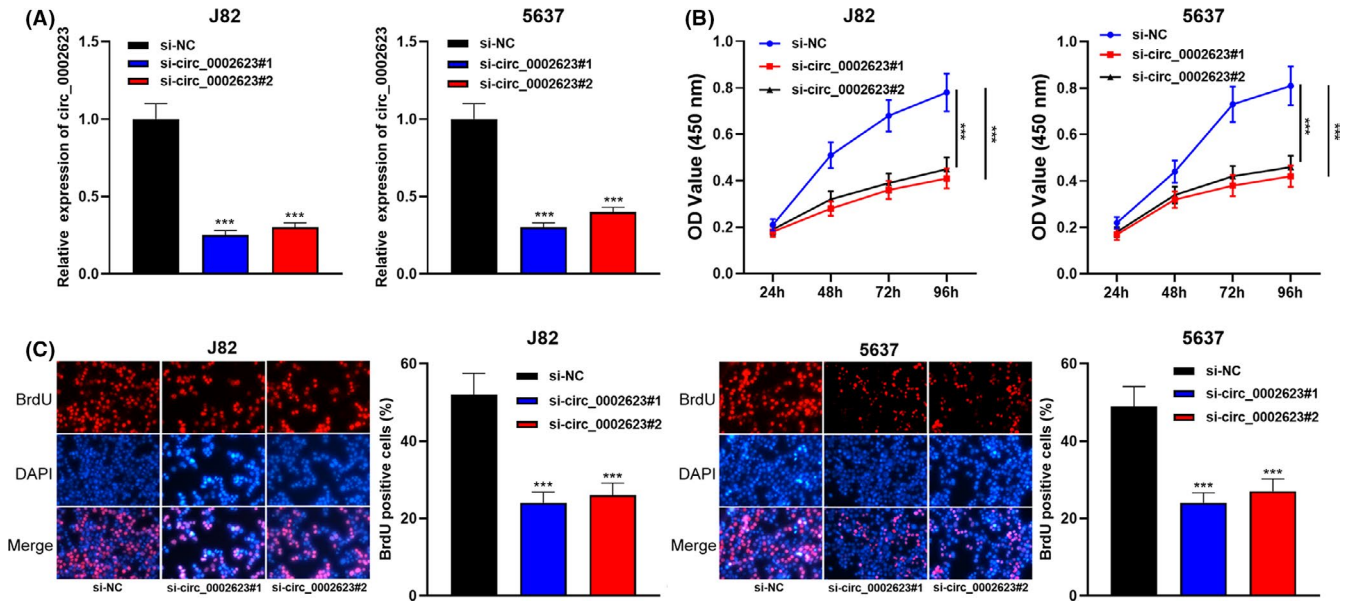

FIGURE 3 Circ_0002623 knockdown inhibits bladder cancer (BCa) cell proliferation, migration, and cell cycle. A, Circ_0002623 knockdown plasmids (si-circ_0002623#1 or si-circ_0002623#2) and the corresponding control plasmids (si-NC) were transfected into J82 and 5637 cells, respectively, and the transfection efficiency was detected by qRT-PCR. B and C, Cell Counting Kit-8 and BrdU assays were used to detect the proliferation ability of BCa cells after transfection. $^{**}p < .01$ and $^{***}p < .001$

circ_0002623 and miR-1276 expression levels were negatively correlated (Figure 5H). Collectively, these data suggest that circ_0002623 binds with miR-1276 to inhibit its expression. It was hypothesized that circ_0002623 regulated BCa progression through miR-1276. Next, miR-1276 inhibitors were transfected into J82 cells with circ_0002623 knockdown, and through CCK-8 assay, BrdU assay, Transwell assay, and flow cytometry it was found that the cotransfection of miR-1276 inhibitors counteracted the biological functions of circ_0002623 knockdown on J82 cells (Figures 6A-E; S3A-E).

3.5 | SMAD2 is a target of miR-1276

MiRNA can bind to mRNA 3'UTR and inhibit its translation.[16] Subsequently, three databases StarBase (http://starbase.sysu.edu.cn/index.php), Tragetscan (http://www.targetscan.org/vert_72/), and Mirdb (http://www.mirdb.org/) were searched for comprehensively analyzing the potential target genes of miR-1276, of which 449 potential target genes were obtained (Figure 7A). Kyoto encyclopedia of genes and genomes (KEGG) analysis showed that the potential target genes of miR-1276 were enriched in various signaling pathways or biological processes such as the Wnt signaling pathway, apoptosis, and tumorigenesis (Figure 7B). SMAD2 was selected for further research, and dual-luciferase reporter gene vectors were constructed based on the binding site between miR-1276 and SMAD2 3′UTR (Figure 7C). Dual-luciferase reporter gene assay showed that miR-1276 overexpression could reduce the luciferase activity of the cells cotransfected with SMAD2-wt but had no significant effect on the luciferase activity of the cells transfected with SMAD2-mut (Figure 7D). MiR-1276 mimics could markedly suppress SMAD2 expression at both mRNA level and protein level (Figure 7E,F). Furthermore, qRT-PCR showed that SMAD2 expression in BCa tissues was significantly higher compared with that in paracancerous tissues. Also, the GEPIA2 database (http://gepia2.cancer-pku.cn/) suggested that high SMAD2 expression was significantly associated with shorter overall survival of BCa patients (Figure 7G,H).

3.6 | Circ_0002623 promotes BCa progression in vivo

The in vitro experiments indicated that circ_0002623 promoted BCa progression. We further explored whether circ_0002623 promoted BCa progression in vivo. T24 cells with stable circ_0002623 overexpression were established. Nude mice were subcutaneously injected with T24 cells with circ_0002623 overexpression, and the tumor growth was monitored. It was revealed that, overexpression of circ_0002623 increased the tumor size and weight (Figure 8A,B). Lung metastasis assay indicated that circ_0002623 overexpression significantly promoted the pulmonary metastasis of T24 cells (Figure 8C, Table 3). Notably, after SMAD2 had been silenced, the promoting effect of circ_0002623 overexpression on the metastasis of T24 cells was reversed; however, the depletion of SMAD2 did not counteract the effects of circ_0002623 overexpression on tumor growth (Figure 8A-C, Table 3).

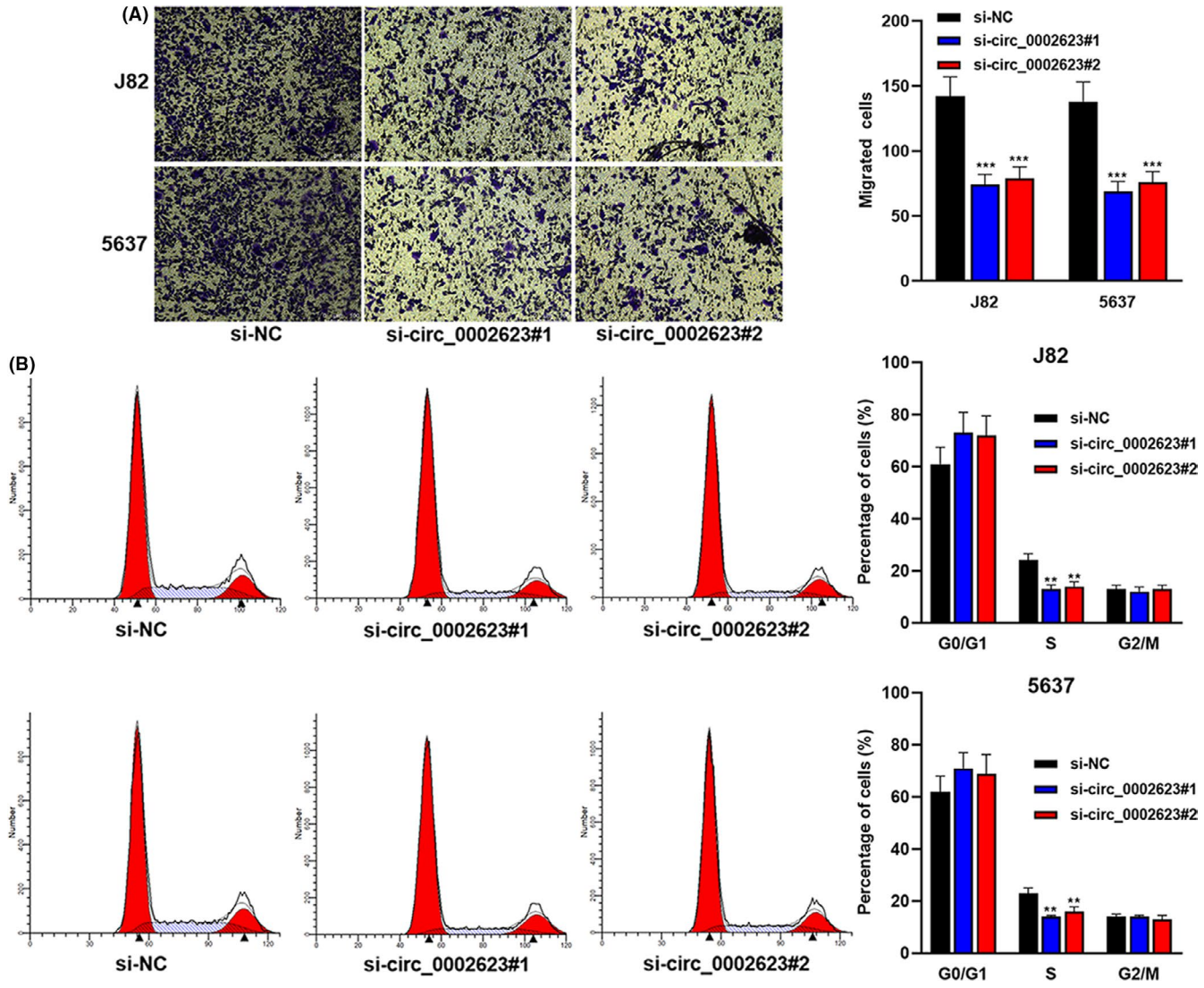

FIGURE 4 Circ_0002623 knockdown inhibits bladder cancer (BCa) cell migration and cell cycle progression. A, Transwell assay was used to detect the migration ability of BCa cells after transfection. B, Flow cytometry was used to detect the cell cycle distribution of BCa cells. $^{**}p < .01$ and $^{***}p < .001$

3.7 | Circ_0002623 promotes the activation of the TGF-β and Wnt signaling pathways by regulating miR-1276/SMAD2

To further clarify the molecular mechanism by which circ_0002623 regulates BCa progression, Gene set enrichment analysis (GSEA) was carried out based on mRNA expression data from the cancer genome atlas (TCGA), and it was revealed that SMAD2 high expression was positively correlated with the activation of the Wnt pathway and TGF-β pathway in BCa (Figure 9A). Western blotting suggested that the expression levels of TGF-β, WNT1, and SMAD2 in T24 cells with circ_0002623 overexpression were significantly increased, and this effect could be reversed by the transfection of miR-1276 mimics or SMAD2 siRNAs (Figure 9B). These results suggested that circ_0002623 could probably regulate Wnt signaling via the miR-1276/SMAD2 axis.

4 | DISCUSSION

In tumorigenesis and cancer progression, circRNAs exert their biological function via different mechanisms. Circular RNAs modulate gene expression through functioning as a scaffold in the assembly of protein complexes, regulating parental gene expression, modulating alternative splicing and RNA-protein interaction, and sponging miRNAs.[17-20] In breast cancer, circTADA2A-E6 suppresses cancer cell growth, migration, invasion, and clonogenicity, thus exerting cancer-suppressing effects; circTADA2A-E6 functions as a molecular sponge of miR-203a-3p to restore the expression of SOCS3.[21] Circ-ITCH suppresses BCa development via sponging miR-17/miR-224 and modulating the expression levels of p21 and PTEN.[19] In the present study, it was revealed that circ_0002623 expression in BCa was markedly elevated, and high circ_0002623 expression was significantly associated with BCa patients' poor prognosis. Functional

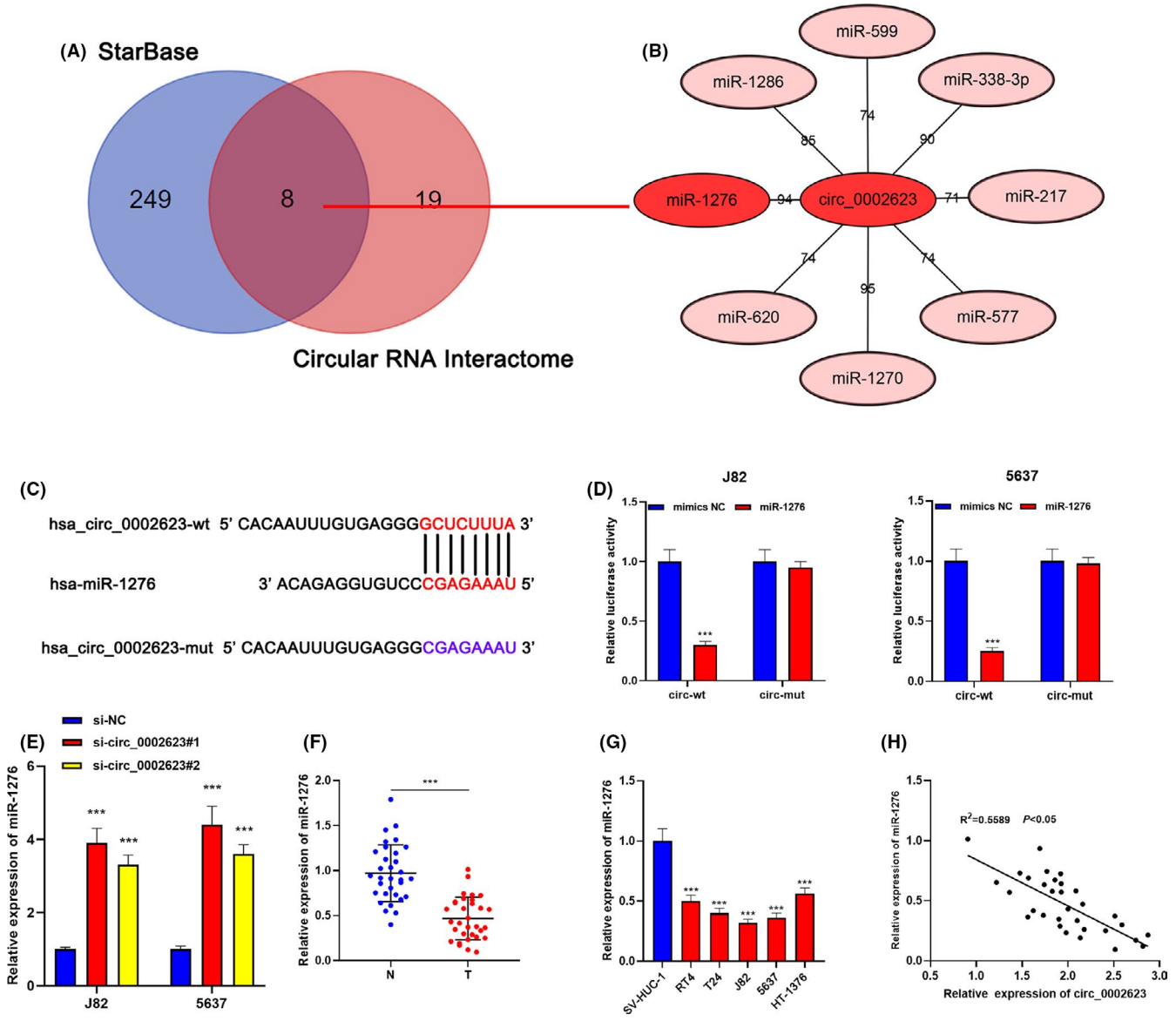

FIGURE 5 Circ_0002623 functions as a competitive endogenous RNA (ceRNA) and sponges miR-1276. A, The Circular RNA Interactome and StarBase databases were used to predict the candidate microRNA (miRNAs) with complementary sequences to circ_0002623. B, Gene-gene interaction network diagram shows eight potential target miRNAs of circ_0002623. C, The binding site between circ_0002623 and miR-1276 was predicted by bioinformatics analysis. D, Dual-luciferase reporter gene assay validated the binding site between circ_0002623 and miR-1276. E, Quantitative RT-PCR was performed to detect the regulatory effect of circ_0002623 knockdown on miR-1276 expression. F and G, Quantitative RT-PCR was performed to detect miR-1276 expression in bladder cancer (BCa) tissues and cell lines. H, Pearson's correlation analysis was used to analyze the correlation between circ_0002623 expression and miR-1276 expression in BCa samples. $^{***}p < .001$

experiments confirmed that knocking down circ_0002623 could significantly restrain BCa cell proliferation and migration, while its overexpression facilitates the malignant phenotypes of cancer cells. These data imply that circ_0002623 is a novel oncogenic factor in BCa and has the potential to be a new diagnostic biomarker and therapy target.

MicroRNAs modulate multiple biological processes, including cell cycle checkpoints, cell proliferation, apoptosis, etc.[22] The abnormal expression of miRNAs is strongly associated with tumor development. For example, miR-125a-5p inhibits BCa cell migration, invasion, and epithelial-mesenchymal transition (EMT) by targeting FUT4.[23] MiR-190b enhances BCa cell multiplication, migration, invasion, and angiogenesis.[24] Some previous studies have reported the biological function of miR-1276 in some malignancies and that it plays a role as an oncomiR or tumor suppressor depending on cancer type. For instance, miR-1276 suppresses gastric carcinoma progression by targeting CTNNB1 and repressing the Wnt pathway.[11] On the contrary, miR-1276 negatively modulates LACTB to accelerate colon carcinoma cell multiplication, migration, invasiveness, and EMT and to reduce autophagy and apoptosis.[25] To our best knowledge, the

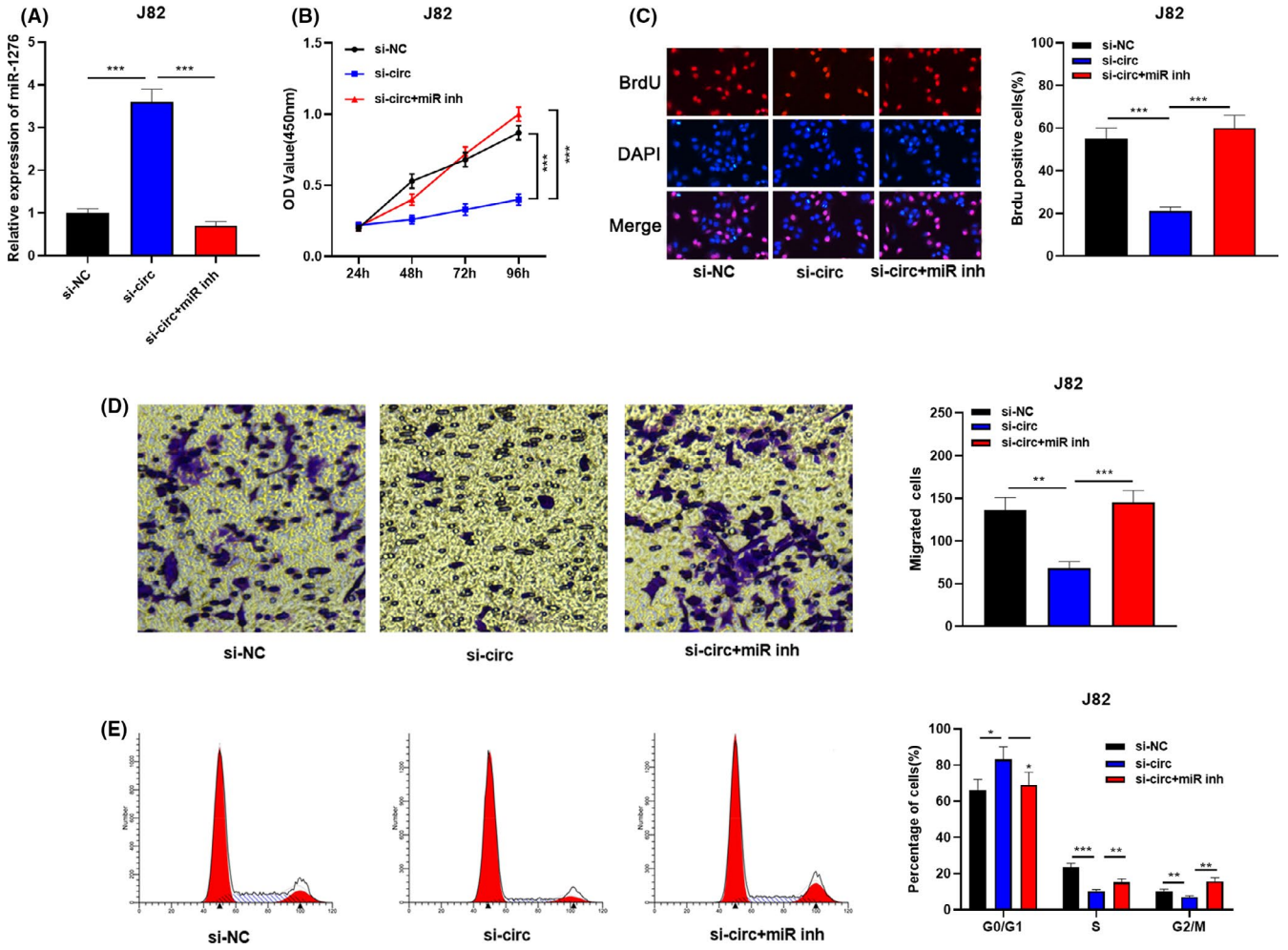

FIGURE 6 Circ_0002623 promotes bladder cancer (BCa) progression by targeting miR-1276. A, si-circ_0002623#1 and miR-1276 were cotransfected into J82 cells, and the transfection efficiency was detected by qRT-PCR. B and C, Cell Counting Kit-8 and BrdU assays were used to detect the proliferation ability of BCa cells after transfection. D, Transwell assay was used to detect the migration ability of BCa cells after transfection. E, Flow cytometry was used to detect the cell cycle distribution of BCa cells. $^{**}p < .01$ and $^{***}p < .001$

expression characteristics and biological functions of miR-1276 in BCa were unclear previously. In the present work, it was revealed that miR-1276 was lowly expressed in BCa tissues and cell lines. Additionally, miR-1276 was identified as the target of circ_0002623, and miR-1276 inhibitors could counteract the inhibiting effect of knocking down circ_0002623 on BCa. Additionally, SMAD2 was identified as a target gene of miR-1276. These data, for the first time, support that miR-1276 has tumor-suppressive properties.

Known as a member of the SMAD family, SMAD2 serves as an intracellular signal transducer and downstream transcriptional regulator of transforming growth factor-β (TGF-β). SMAD2 has been reported to be implicated in fetal development, inflammatory responses, tissue differentiation, and even in tumorigenesis.[26] TGF-β members are known as vital cytokines regulating embryogenesis and tissue homeostasis through transmembrane TGF-β type II (TβR II) and type I (TβR I) and serine/threonine kinase receptors. Reportedly, abnormal activation of the TGF-β/SMAD pathway promotes the progression of various human cancers, including lung cancer, breast carcinoma, and renal cell carcinoma.[27,28] Some previous studies report that the TGF-β/SMAD pathway can be modulated by circRNAs in BCa. Specifically, it is reported that circRIP2 accelerates BCa progression via the miR-1305/TGF-β2/smad3 pathway.[29] Interestingly, in the present work, it was revealed that circ_0002623 could regulate SMAD2 via repressing miR-1276, which further validated the importance of circRNA in modulating the activation of the TGF-β/SMAD pathway. It is worth noting that the TGF-β/SMAD pathway is reported to promote the metastatic potential of cancer cells, while it represses proliferation.[30] However, in the present work, it was observed that circ_0002623 promoted not only the migration of BCa cells but also their proliferation, and these data suggest that circ_0002623 had another unknown downstream mechanism to promote BCa progression.

Wnt/β-catenin signaling is one of the main oncogenic pathways.[31] Wnt and its downstream effectors regulate various processes during cancer progression (including tumorigenesis, tumor growth, cellular senescence, and cellular death), differentiation, and

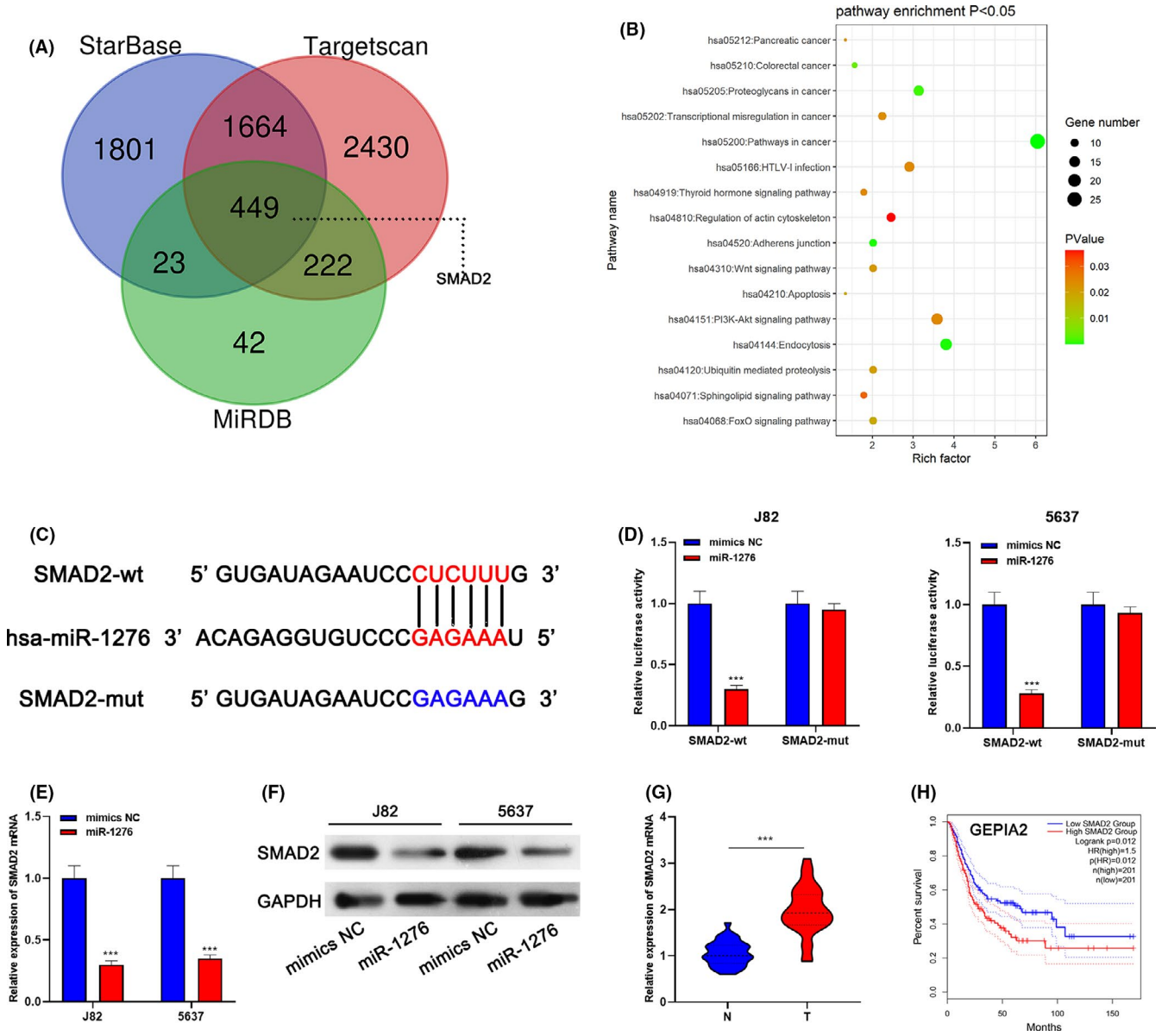

FIGURE 7 SMAD2 is a target of miR-1276. A, Three databases, StarBase, TargetScan, and miRDB, were used to comprehensively analyze the potential target genes of miR-1276. B, KEGG analysis of the signaling pathways where miR-1276 target genes are enriched. C, The binding site between miR-1276 and SMAD2 was predicted by bioinformatics analysis. D, Dual-luciferase reporter gene assay validated the binding site between miR-1276 and SMAD2. E and F, Quantitative RT-PCR and Western blot were used to detect the regulatory effect of miR-1276 on SMAD2 expression. G, Quantitative RT-PCR was used to detect SMAD2 expression in 32 pairs of bladder cancer (BCa) tissues and the paracancerous tissues. H, The GEPIA2 database was used to analyze the relationship between SMAD2 expression and BCa patients' overall survival. ***$p < .001$

metastasis.[32] Some previous studies have reported that there is a cross talk between the Wnt/β-catenin pathway and TGF-β/SMAD pathway.[33,34] Interestingly, in the present work, we demonstrated that SMAD2 could positively regulate the expression of WNT1 in BCa cells, and circ_0002623 could regulate WNT1 expression via the miR-1276/SMAD2 axis. However, the detailed mechanism by which SMAD2 regulates WNT1 is still obscure, which requires further investigation.

To sum up, this study shows that circ_0002623 expression is increased in BCa, and miR-1276 expression is decreased. Mechanistically, circ_0002623 upregulates SMAD2 expression by sponging miR-1276, thus facilitating the malignant phenotype of BCa cells (Figure 10). Our study provides novel insights into BCa diagnosis and treatment. However, there are several limitations to the present study. Firstly, as mentioned above, circ_0002623 may target other miRNAs and modulate other downstream pathways to exert its biological functions, and the underlying mechanism of circ_0002623 requires further investigation. Additionally, the regulatory function of circ_0002623 on miR-1276 and SMAD2 is only investigated with in vitro models.

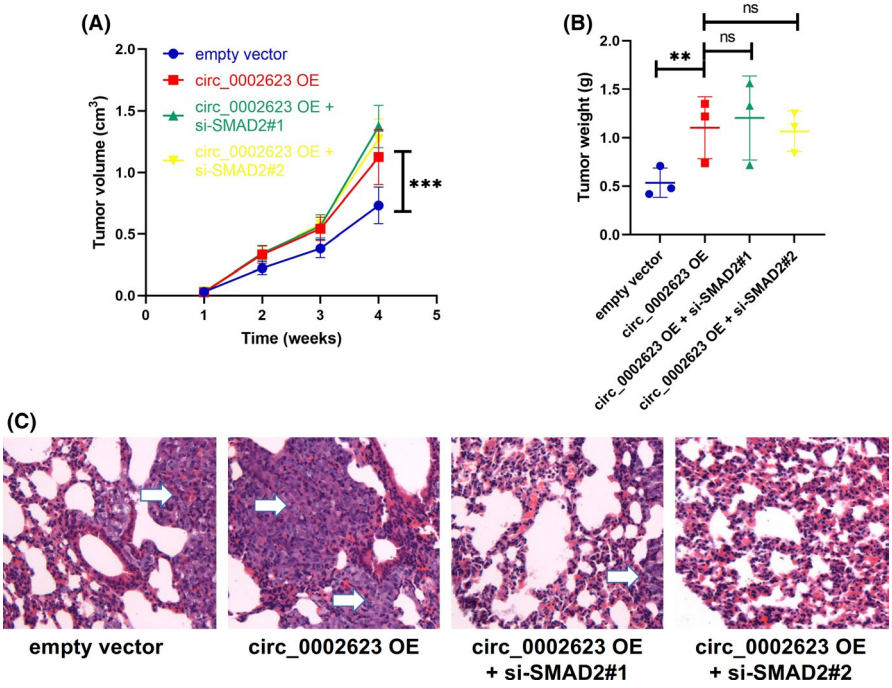

FIGURE 8 Circ_0002623 promotes bladder cancer (BCa) progression in vivo. A and B, After T24 cells had been inoculated subcutaneously, the tumor volume and weight of the mice were measured (n = 3 in each group). C, HE staining was used to detect the lung metastasis of BCa after the nude mice had been injected with T24 cells through the caudal vein (n = 10 in each group). The arrows indicate the metastatic nodules. OE, overexpression. $^{**}p < .01$ and $^{***}p < .001$. ns, no significance

TABLE 3 The pathological evaluation of the metastatic nodules in the lung tissues of the nude mice

Groups	Severe metastasis	Moderate metastasis	Mild metastasis or no metastasis	*p* value
The control (n = 10)	1	4	5	
circ_0002623 OE (n = 10)	7	2	1	vs the control group: 0.037*
circ_0002623 OE +si-SMAD2#1 (n = 10)	2	1	7	vs circ_0002623 OE group: 0.022*
Circ_0002623 OE +si-SMAD2#2 (n = 10)	1	3	6	vs circ_0002623 OE group: 0.018*

Abbreviations: OE, overexpression; si-SMAD2, SMAD2 siRNA.

*$p < .05$, Fisher's exact test.

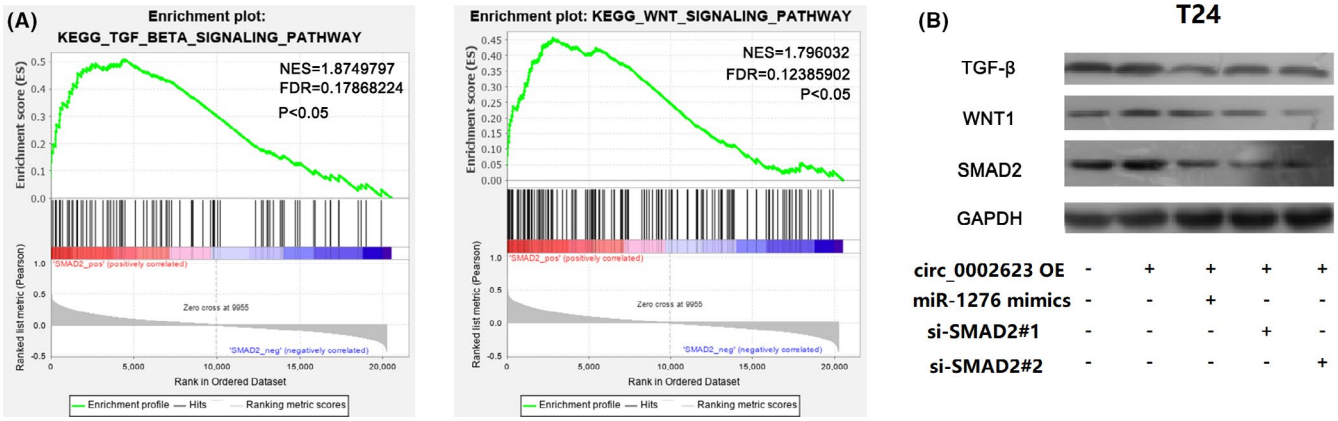

FIGURE 9 Circ_0002623 promotes the activation of the TGF-β and Wnt signaling pathways by regulating SMAD2. A, Gene set enrichment analysis (GSEA) was performed to predict the biological effects of SMAD2 in bladder cancer (BCa) tissues, and it showed that SMAD2 high expression was associated with the activation of the TGF-β and Wnt signaling pathways. B, Western blotting was used to detect the regulatory effects of circ_0002623, miR-1276, and SMAD2 on the expression levels of TGF-β and WNT1

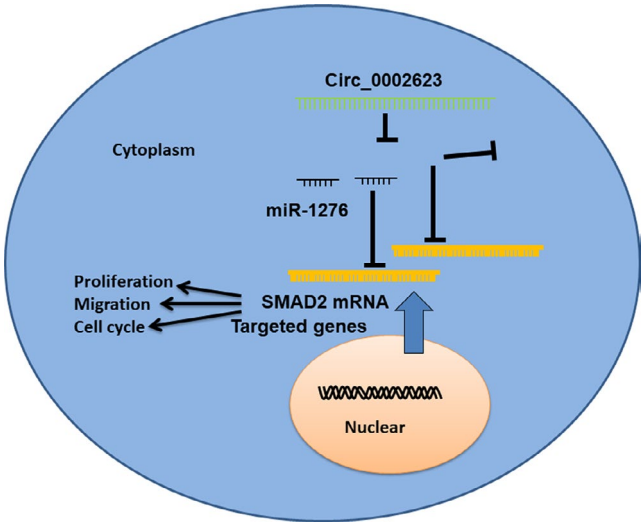

FIGURE 10 Graphic abstract: circ_0002623 increases SMAD2 expression via sponging miR-1276, thereby promoting the malignant phenotypes of bladder cancer (BCa) cells

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DISCLOSURE

The authors declare that they have no competing interests.

ETHICAL APPROVAL

Our study was approved by the Ethics Review Board of Qilu Hospital.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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