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ORIGINAL RESEARCH

RETRACTED ARTICLE: Circular RNA *circ-Foxo3* induced cell apoptosis in urothelial carcinoma via interaction with *miR-191-5p*

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Background: Circular RNAs (circRNAs) play a critical role in cancer. Emerging evidence has shown circ-Foxo3, a circRNA, was dysregulated in a variety of tumor types. However, the exact role of *circ-Foxo3* in bladder cancer has never been studied.

Methods: We measured the expression level of circ-Foxo3 in human and murine bladder cancer tissues and in various human bladder cancer cell lines. We induced bladder cancer in mice by a carcinogen N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN). circ-Foxo3 was overexpressed in mice by lentiviral gene transfer and in cultured cells via overexpression plasmid. The effect of circ-Foxo3 on apoptosis was examined via apoptotic marker staining, Western blot, and flow cytometry. We further characterized the interaction between circ-Foxo3 and miR-191 and its functional impact on bladder cancer cells.

Results: circ-Foxo3 was downregulated in bladder cancer in vivo and in vitro, and was upregulated in response to apoptotic stress. Overexpression of circ-Foxo3 promoted bladder cancer cell apoptosis in BBN induced and in human bladder cancer cell lines. miR-191-5p suppressed circ-Foxo3 expression and the pro-apoptotic effect of *circ-Foxo3* in bladder cancer cells via direct targeting the 3′-untranslated region (3′-UTR) of *circ-Foxo3*.

Conclusion: circ-Foxo3 was downregulated in bladder cancer in vivo and in vitro, and promoted bladder cancer apoptosis via direct interaction with miR-191. circ-Foxo3 could be a potential therapeutic target for bladder cancer.

Keywords: bladder cancer, circular RNA, apoptosis, circ-Foxo3, miR-191

Background

Bladder cancer is the 9th most commonly diagnosed cancer worldwide and the 4th most common cancer in men.[1,2] Globally, about 430,000 new bladder cancer cases are diagnosed annually.[1] In the United States, over 80,000 new cases of bladder cancer are diagnosed each year, and over 17,000 patients die from bladder cancer annually.[2] Urothelial carcinoma, also known as transitional cell carcinoma, is the most common type of bladder cancer. Urothelial carcinoma originates from the urothelial cells that line the inner layer of urinary tract. The development of urothelial carcinoma is predisposed by certain risk factors, including smoking and exposure to certain industrial carcinogens such as aromatic amines. To date, there is no definitive cure to urothelial carcinoma, and its mortality rate has maintained stable. Therefore, identifying early diagnostic markers and novel therapeutic targets for bladder cancer, particularly urothelial carcinoma, remains a major public health need.

Circular RNA (circRNA) is a type of single-stranded RNA that forms a closed loop by joining the 5′ and 3′ ends of a linear RNA. CircRNAs are ubiquitously expressed from archaea to eukaryotes and are evolutionary conserved, strongly

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suggesting their functional importance.[3] CircRNA can be protein-coding or non-coding.[4,5] Although the detailed function of most circRNAs remains unclear, one major role of those non-coding circRNAs is shown to be gene regulation,[6] potentially by interaction with microRNAs or RNA-binding proteins.[7,8]

circ-Foxo3 is a circRNA derived from the *FOXO3* gene that also encodes the linear *FOXO3* mRNA.[9] The expression of *circ-Foxo3* appeared independent of the expression of *FOXO3* mRNA, and may have regulatory function on targets beyond the linear *FOXO3* mRNA.[10] Recently, emerging evidence has indicated that *circ-Foxo3* was detectable in multiple cancers, and that *circ-Foxo3* was associated with cell cycle retardation or apoptosis.[10–14] However, the role of *circ-Foxo3* in bladder cancer has yet to be fully understood.

Here, we explored the role of *circ-Foxo3* in bladder cancer. We found *circ-Foxo3* was dysregulated in bladder cancer tissue in vivo and in vitro. *circ-Foxo3* was a direct target of the microRNA *miR-191-5p*. Together, a *miR-191-circ-Foxo3* axis appeared critical to bladder cancer apoptosis.

Methods

Human bladder cancer samples

The study involving human samples was approved by the Institutional Review Board of The First Affiliated Hospital of Harbin Medical University. Written informed consent was obtained from all patients upon recruiting. Thirty pairs of fresh bladder cancer tissues and adjacent normal bladder tissue were excised during partial or radical cystectomy from patients with confirmed diagnosis of urothelial carcinoma at the Department of Urology of The First Affiliated Hospital of Harbin Medical University between 2016 and 2017. None of the patients had medication or radiation therapy prior to surgery. The specimens were snap-frozen in liquid nitrogen immediately after excision. Pathological and histological diagnoses were performed by two pathologists independently, and the diagnosis of urothelial carcinoma was confirmed in all samples. The grades and stages of specimens were classified using 2004 World Health Organization Consensus Classification and Staging System.

Cell culture and treatment

Three cell lines of human transitional cell carcinoma, T24, UM-UC-3 and J82, and a normal human uroepithelial cell line SV-HUC-1, were obtained from the American Type Culture Collection (ATCC, USA). All cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco, USA). Cells were kept in an incubator at 37°C with humidified atmosphere containing 5% CO_2. Medium was replaced every 2–3 days.

Cells receiving transfection were treated in RPMI-1640 basal medium (serum-free) containing 0.4 mM hydrogen peroxide (H_2O_2), or 1 μg/mL doxorubicin (DOX), or 2 μg/mL cisplatin (CP) for 24 h.

Bladder tumor model in mice

All animal protocols were approved by the Ethical Committee of The First Affiliated Hospital of Harbin Medical University and adhered to the Guide for the Care and Use of Laboratory Animals (NRC 2011, 8th Edition). Male, 8-week-old C57BL/6 mice were obtained from The First Affiliated Hospital of Harbin Medical University. Mice were housed in a temperature-controlled environment with 12-h light/dark cycles and unrestricted access to food and drinking water. To induce carcinogenesis in the bladder, 0.05% N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN; Tokyo Chemical Industry Co Ltd, Japan) was added to the drinking water for 17 weeks until tissue harvesting. BBN-free water (vehicle) was added in the drinking water in equal volume for 17 weeks as control treatment. The induction of bladder cancer was histologically confirmed at tissue harvesting.

Histology

Mice were euthanized by CO_2 inhalation. The bladders were exposed. A small fraction of bladder tumor tissue and the adjacent normal bladder tissue was removed for RNA extraction. The remaining bladder was inflated and soaked in 4% formaldehyde in phosphate-buffered saline (PBS) overnight. Fixed bladders were cut in half, positioned in cassettes, soaked in 70% alcohol overnight, and embedded in paraffin. The paraffin-embedded blocks were cut into 6-μm-thick serial sections, stained with hematoxylin and eosin (H&E), and gold enhanced for visualization by light microscopy.

Circrna vectors

The *circ-Foxo3* over-expression plasmid was constructed by sub-cloning the human *circ-Foxo3* cDNA (synthesized by TSINGKE, China) onto a pCD-ciR circRNA expression vector (Geneseed Biotech, China), which has a front circular frame and a back circular frame to ensure functional circRNA transcription. The sequence-verified plasmid was transfected into cells using Lipofectamine 2000

(Thermo Fisher Scientific, USA). The empty pCD-ciR vector was used as control.

The control (circControl-GFP) and *circ-Foxo3* overexpression (circFoxo3-GFP) lentiviruses were generated by Hanbio (China). For somatic gene transfer, circControl-GFP or circFoxo3-GFP lentivirus (2×10^8 viral genome copies/μL) was injected into the male 8-week-old C57BL/6 mice (5 μL each). Mice were divided into the following 4 groups for treatments at 2 weeks after lentiviral injection: circControl + vehicle, circFoxo3+ vehicle, circControl + BBN, and circFoxo3+ BBN.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from homogenized tissue or cultured cells using an RNeasy Mini Kit (Qiagen, USA) following the manufacturer's instruction. cDNA was synthesized using PrimeScript RT Master Mix (Takara Bio, China) with 2 μg total RNA input in 20 μL reaction. A portion of the reverse transcription product (1 μL, equal to 0.1 μg cDNA) was saved for regular PCR with two appropriate primers. qPCR was performed using the miScript SYBR Green PCR Kit (Qiagen, USA) with 1 μL cDNA template. The relative expression of RNA was quantified using the ΔΔCq method.[15]

Apoptosis assay

Cells were grown on 6-well plates after transfection. Apoptosis was quantified using the Annexin V-FITC apoptosis kit (Thermo Fisher Scientific, USA). Briefly, cells were detached 48 h post-transfection, washed with PBS, and resuspended in 500 μL binding buffer containing 5 μL propidium iodide (PI) and 5 μL Annexin V-FITC. After incubation at room temperature for 10 min, cells were washed and resuspend in binding buffer. Apoptosis was analyzed by a FACSCalibur flow cytometer (BD Biosciences, USA).

Western blot

Cells were harvested in RIPA lysis buffer and protein concentration was normalized by the Brandford protein assay. Normalized protein was boiled in Laemmli sample buffer, and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein was transferred onto polyvinylidene fluoride membrane and immunoblotted as described.[16] The following primary antibodies were used: anti-cleaved-caspase3 (1:1000), anti-Bcl2 (1:1000), anti-Bax (1:1000), and anti-GAPDH (1:1000) (all from Abcam, UK).

Cell viability assay

Cell viability was measured by the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) following the manufacturer's protocol. Briefly, transfected cells were plated on 96-well plates and cultured in 100 μL/well medium for 24 h. CCK-8 solution was added to the plates (10 μL/well) at 0 h, 24 h, 48 h, 72 h, and 96 h, respectively, and further incubated for 4 hrs for colorimetric reaction. Absorbance at 450 nm was measured with a microplate reader to represent cell viability.

Luciferase reporter assay

The 3′-untranslated region (3′-UTR) of *circ-Foxo3* RNA containing the putative *miR-191* binding sequence was amplified by PCR and cloned into a pmirGLO (Promega, USA) dual-luciferase miRNA target expression vector (named pmirGlo-CREB1-WT; WT). Targeted mutation of the vector was generated within the *circ-Foxo3* 3′-UTR sequence by a QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies, USA) resulting a mutant luciferase reporter vector (named pmirGlo-CREB1-MUT; MUT). For the luciferase reporter assays, WT or MUT luciferase reporter vectors were transfected into J82 cells on 6-well plates in combination with *miRNA-191* mimics or non-targeting control microRNA mimics using Lipofectamine 2000 (Thermo Fisher Scientific, USA). The firefly luciferase activity was measured 48 h post-transfection using a dual-luciferase reporter assay system (Promega, USA) following the manufacturer's instruction, and normalized to *Renilla* luciferase activity.

Terminal deoxynucleotidyl transferase dutp nick end labeling (TUNEL) assay

Apoptotic DNA fragmentation in situ was detected by the TUNEL assay as previously described.[17]

Statistical method

All data were expressed as mean ± standard deviation (SD). Difference of means between two groups was compared by two-tailed Student's *t*-test, with significance set as $p < 0.05$. Difference of means among three or more groups was analyzed by one-way analysis of variance (ANOVA), followed by multiple comparisons test, and multiplicity adjusted $p < 0.05$ was considered statistically significant.

Results

circ-Foxo3 was downregulated in bladder cancer in vivo and in vitro

We first examined the expression of *circ-Foxo3* in bladder cancer tissues and cell lines. RT-qPCR showed the RNA expression of *circ-Foxo3* was significantly downregulated in tumor tissues isolated from bladder cancer patients as compared with the adjacent normal bladder tissues (Figure 1A). *circ-Foxo3* expression was also reduced in murine bladder tumors induced by 17-week oral treatment of BBN, a carcinogen that effectively induces bladder cancer (Figure 1B).

We also measured *circ-Foxo3* expression in bladder cancer in vitro. The expression of *circ-Foxo3* RNA was significantly down-regulated in human bladder cancer cell lines T24, UM-UC-3, and J82, as compared with the immortalized normal bladder cell line SV-HUC-1 (Figure 1C).

circ-Foxo3 was upregulated in response to apoptotic stress

Next, we explored the role of *circ-Foxo3* in apoptosis. We first tested the level of apoptosis in J82 cells treated with doxorubicin, cisplatin or H_2O_2. Western blot showed t apoptotic protein markers was markedly increased followin these treatments, as indicated by increased cleaved-caspase3 expression and increased ratio of the pro-apo otic p otein Bax over the pro-survival protein Bcl2 (Fig e 2A).

We further characterized the apoptot stres in ditional cell lines. The expression of *circ- o3* was si ficantly upregulated in bladder cancer ce lines 24, UM-UC 3 and J82 as well as the normal l dder epithe l cell line SV-HUC-1 upon treatment th doxo ubicin (Figure 2B), cisplatin (Figure 2C), and H_2O_2 (gure 2D).

circ-Foxo3 induced apoptosis in bladder tumor cells of BBN mice and in bladder cancer cell lines

To test the effect of *circ-Foxo3* on apoptosis in vivo, we injected control (circControl-GFP) or *circ-Foxo3* (circFoxo3-GFP) lentiviral vectors for somatic gene transfer in mice. We then treated mice with oral BBN for 17 weeks to induce bladder cancer. Somatic gene transfer of *circ-Foxo3* significantly increased cell apoptosis in the bladder tissue of BBN mice as measured by TUNEL staining (Figure 3A). Likewise, We ern b showed that the expression the cleaved-caspa and the rat of Bax to Bcl2 was increased in mice r ceivi *circ-Foxo* lentiviral injection (Figure 3B).

We then examined e effect *circ-Fo 3* overexpression on apoptosis vit e transi tly overexpressed *circ-Foxo3* RN in human b dder ancer cell lines with a *circ-Foxo3* asmi (Figure 4A). Overexpression of *circ-Foxo3* promoted bla r cancer cell apoptosis, as measured by Western blot o apoptotic markers (Figure 4B) and low cytome y (Figure 4C).

rthermore, e examined the viability of bladder cancer ll li s. Overexpression of *circ-Foxo3* signifi- ly reduced bladder cancer cell viability as measured b the CCK-8 assay (Figure 4D).

miR-191-5p suppressed circ-Foxo3 expression in bladder cancer cells

Our preliminary analysis revealed a strong negative correlation between the expression of *circ-Foxo3* and *miR-191-5p* RNA in 30 tumor biopsies from bladder cancer patients (Figure 5A). *miR-191-5p* is a novel microRNA that was implicated in a variety of solid tumors.[18–24] The strong

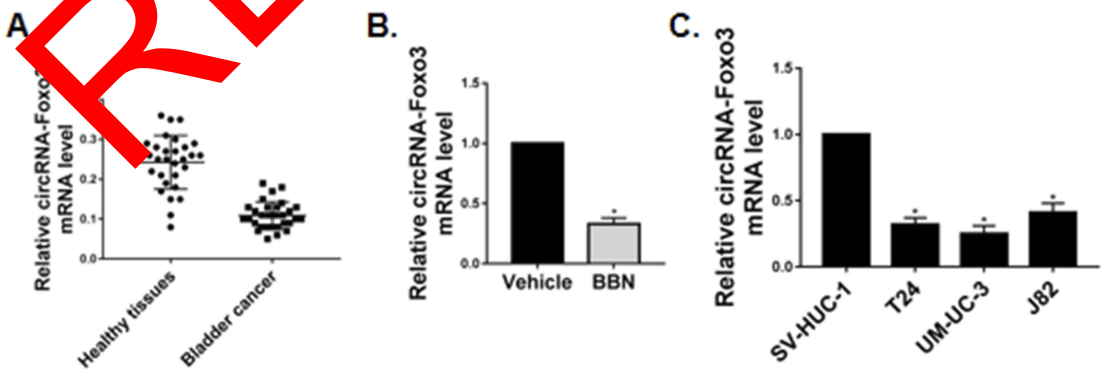

Figure 1 *circ-Foxo3* was downregulated in bladder cancer in vivo and in vitro. (**A**) Expression of *circ-Foxo3* RNA in tumor tissues and adjacent normal bladder tissues taken from bladder cancer patients (n=30). (**B**) Expression of *circ-Foxo3* RNA in tumor tissues and adjacent normal bladder tissues from mice treated with vehicle or BBN for 17 weeks (n=10). (**C**) Expression of *circ-Foxo3* RNA in different bladder cell lines (n=5). Mean ± SD, *p<0.05.

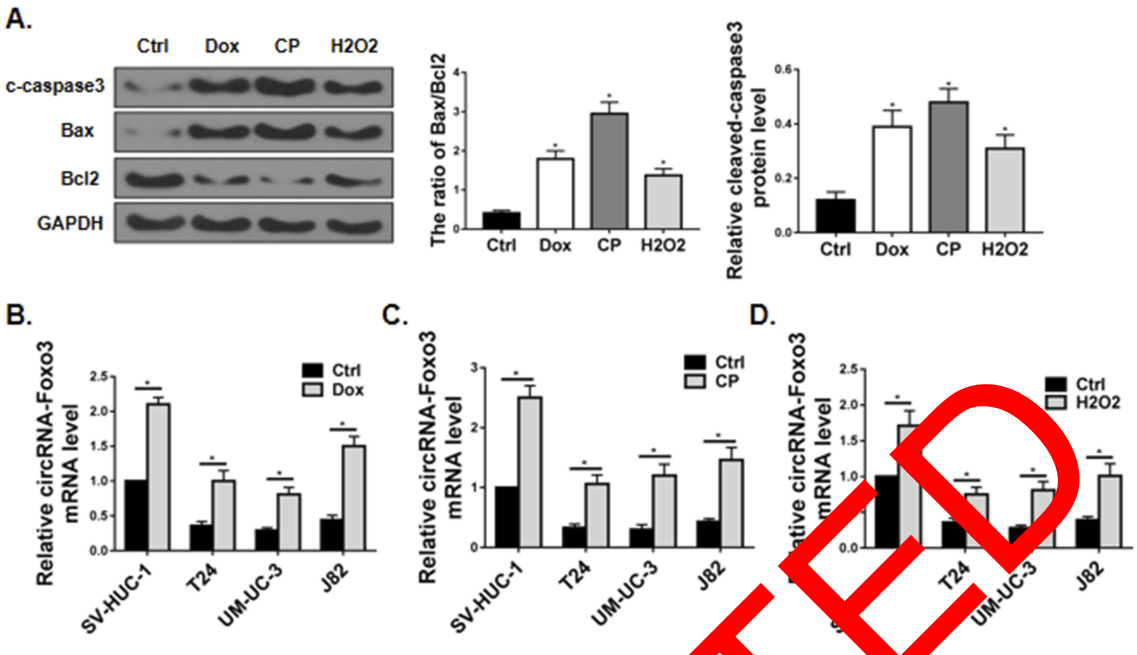

Figure 2 *circ-Foxo3* was upregulated in response to apoptotic stress. (**A**) Western blot of cleaved-caspase3 (c-caspase3), Bax, Bcl2 and GAPDH expression in J82 cells treated with vehicle (Ctrl), doxorubicin (Dox; 1 μg/mL), cisplatin (CP; 2 μg/mL), and H_2O_2 (H2O2; 0.4 mM). The band intensity relative to GAPDH, and the ratio of Bax/Bcl2 band intensity were shown on the right. (**B**–**D**) Expression of *circ-Foxo3* RNA level in bladder cancer cells treated with vehicle (**B**–**D**), doxorubicin (**B**), cisplatin (**C**), and H_2O_2 (**D**), respectively (n=5). Mean ± SD, *p<0.05.

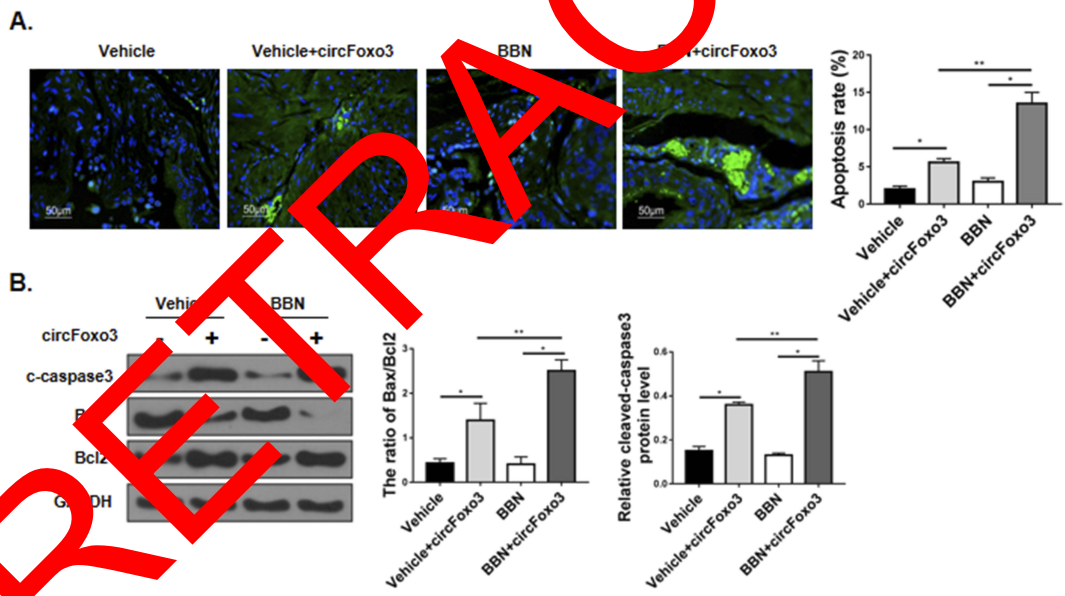

Figure 3 *circ-Foxo3* promoted bladder tumor cell apoptosis in BBN mice. Male, 8-week-old C57BL/6 mice received control (circControl-GFP) and *circ-Foxo3* (circFoxo3-GFP) somatic gene transfer by lentiviral injection, followed by exposure to oral vehicle or BBN treatment for 17 weeks. (**A**) The bladder tumor cell apoptosis was evaluated by the TUNEL assay (left) and quantified (right; n=10). (**B**)Western blot of cleaved-caspase3 (c-caspase3), Bax, Bcl2 and GAPDH expression in murine bladder tissues. The band intensity relative to GAPDH, and the ratio of Bax/Bcl2 band intensity were quantified (n=5). Mean ± SD, *p<0.05, **p<0.01.

correlation between *circ-Foxo3* and *miR-191-5p* RNA expression prompted us to further examine their potential functional interaction in bladder cancer cells.

In an attempt to search for the direct interaction between *circ-Foxo3* and *miR-191-5p* RNA, we identified a predicted *miR-191-5p* target site within the 3′-UTR of *circ-Foxo3* RNA using the TargetScan algorithm (http://www.targetscan.org) (Figure 5B). To experimentally validate the interaction, we designed luciferase reporters coupled with either wild-type (WT) or mutant (MUT) 3′-UTR sequence of *circ-Foxo3*, and

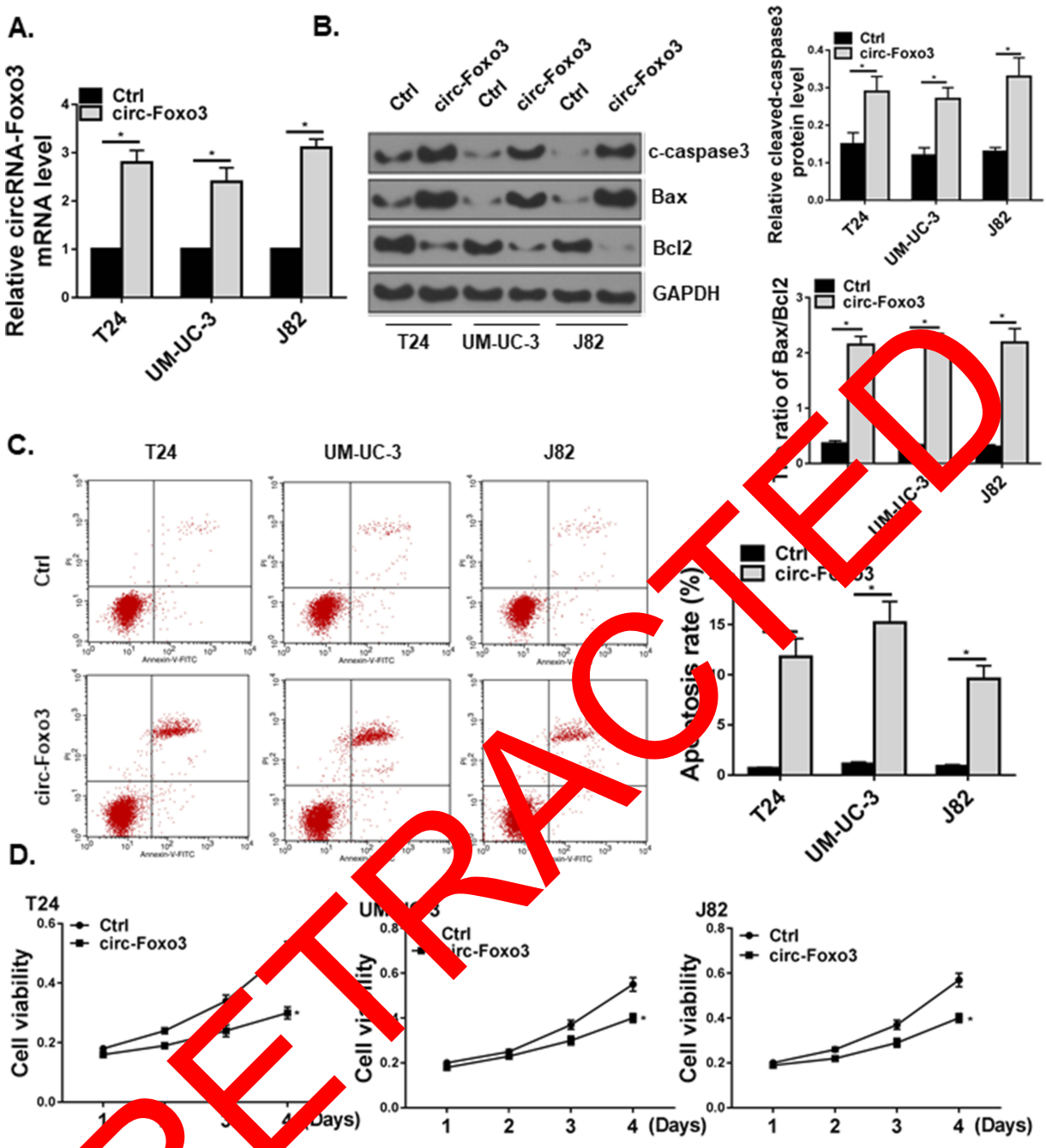

Figure 4 *circ-Foxo3*-induced [illegible] and reduced viability in bladder cancer cells. T24, UM-UC-3, and J82 bladder cancer cell lines received control (Ctrl) or *circ-Foxo3* overexpression (*circ-Foxo3* [illegible]ids. (**A**) Expression of *circ-Foxo3* RNA level in bladder cell lines. (**B**) Western blot of cleaved-caspase3 (c-caspase3), Bax, Bcl2 and GAPDH expression in cells receiving treatment. The band intensity relative to GAPDH, and the ratio of Bax/Bcl2 band intensity were quantified (n=5). (**C**) Analysis of apoptosis in treated bladder cancer cell lines by flow cytometry (n=5). (**D**) Cell viability measured by the CCK-8 assay in T24, UM-UC-3, and J82 bladder cancer cell lines. Mean ± SD, *$p<0.05$.

co-transfected the reporters with a *miR-191-5p* mimic or inhibitor in bladder cancer cells. The expression of *miR-191-5p* was significantly enhanced by *miR-191-5p* mimic and reduced by its inhibitor (Figure 5C). In J82 bladder cancer cells, co-transfection of *miR-191-5p* significantly suppressed wild-type, but not mutant, *circ-Foxo3* luciferase reporter activity (Figure 5D). These results suggested that the 3′-UTR of *circ-Foxo3* was a direct binding target of *miR-191-5p*.

Similarly, ectopic overexpression of *miR-191* significantly suppressed the expression of *circ-Foxo3* in T24, UM-UC-3 and J82 cells, while silencing of *miR-191* significantly enhanced expression of *circ-Foxo3* (Figure 5E). These data

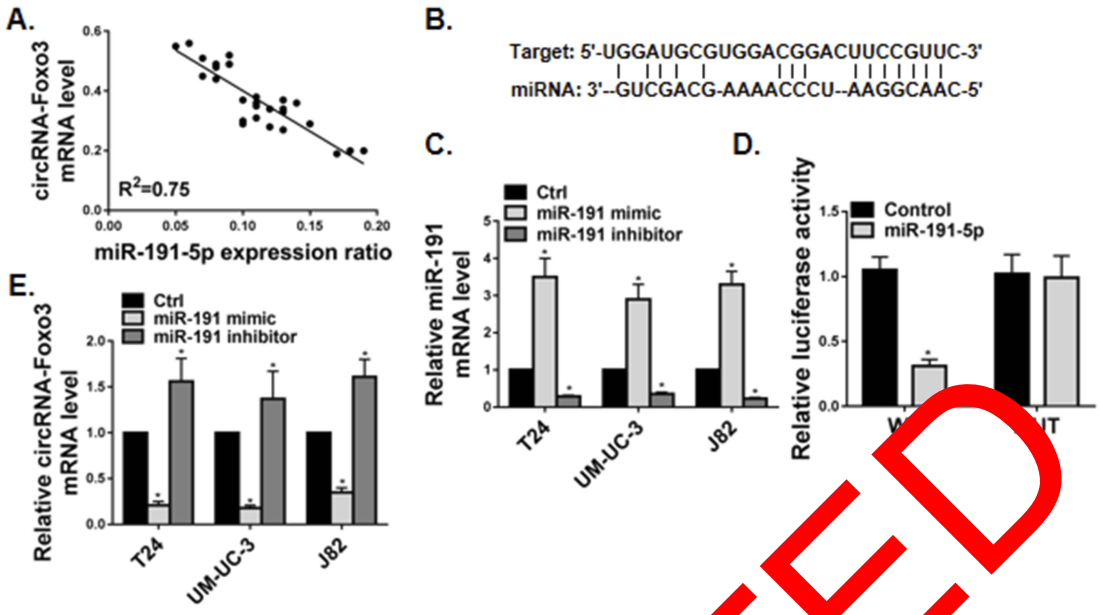

Figure 5 *miR-191-5p* suppressed *circ-Foxo3* expression in bladder cancer cells. (**A**) Correlation betwee[illegible]*-Foxo3* and *miR-*[illegible]RNA expression in tumor biopsy tissue from bladder cancer patients (n=30). (**B**) Bioinformatic analysis using the TargetScan algorithm (http[illegible]www.ta[illegible]scan.org/) predicted a putative *miR-191-5p* target site in the 3′-UTR of *circ-Foxo3*. (**C**) Bladder cell lines were transfected with a non-targeting microRNA (Ctrl), or a *miR-19*[illegible] mimic, or a *miR-191-5p* inhibitor. The RNA expression of *miR-191* was measured by RT-qPCR (n=5). (**D**) *circ-Foxo3* expression luciferase reporter a[illegible]uciferase repo[illegible]vector carrying the wild-type (pmirGlo-CREB1-WT; WT) or mutant (pmirGlo-CREB1-MUT; MUT) 3′-UTR sequence of *circ-Foxo3* were transf[illegible]ed into J82 cells in combina[illegible]on with a *miR-191-5p* mimic (*miR-191-5p*) or a non-targeting microRNA mimic (Control). The relative luciferase activity (arbitrary units, no[illegible]alized to *Renilla* luciferase activity) was analyzed 48 hrs post-transfection. (**E**) The expression of *circ-Foxo3* was measured by RT-qPCR in bladder cancer cells receiving non[illegible]rgeting microR[illegible](Ctrl), or a *miR-191-5p* mimic, or a *miR-191-5p* inhibitor (n=5). Mean ± SD, **p*<0.05.

suggest that transcription of *circ-Foxo3* RNA is suppres[illegible]d by *miR-191* in bladder cancer cells.

miR-191-5p suppressed ap[illegible]ptosis[illegible] inhibiting circ-Foxo3 in [illegible]bladde[illegible] cancer cells

To elucidate the functi[illegible]al relevan[illegible] of the interaction between *miR-191* an[illegible]*circ-Foxo3*, we t[illegible]nsfected bladder cancer cells with [illegible]e *miR-1[illegible]-5p* mimic, and treated cells with cisplatin to ind[illegible] apoptosi[illegible] Western blot showed cisplatin-in[illegible]ced [illegible]poptos[illegible] wa[illegible] significantly attenuated by overex[illegible]ession o[illegible] *miR-191* (Figure 6A). Notably, overexpression o[illegible]*irc-Foxo3* alone also increased apoptosis (Figure 6B). [illegible]-transfection with *miR-191* mimic, however, effectively supp[illegible]ssed apoptosis induced by *circ-Foxo3* as indicated by Western blot (Figure 6B) and flow cytometry (Figure 6C). Consistent with the cell apoptosis measurements, CCK-8 assay showed that ectopic overexpression of *circ-Foxo3* significantly reduced cell viability, which was partially rescued by concurrent transfection of *miR-191* mimic in T24, UM-UC-3 and J82 cells, suggesting a pro-survival effect of *miR-191* on bladder cancer cells that was partially dependent on targeting of *circ-Foxo3* (Figure 6D).

Discussion

The primary finding of our study is that *circ-Foxo3* regulated bladder cancer growth in vivo and in vitro. The expression of *circ-Foxo3* was lower in bladder cancer tissues and bladder cancer cell lines, and was upregulated in response to apoptotic stress. Overexpression of *circ-Foxo3* promoted bladder cancer apoptosis in vivo and in vitro, which was partially attributable to the direct targeting of *circ-Foxo3* by *miR-191*.

Our study is the first to identify a critical role of a circRNA in bladder cancer growth. Several recent studies reported that *circ-Foxo3* regulated progression and proliferation of breast cancer,[11,25] lung cancer,[12] and gastric cancer.[26] Our current data showed *circ-Foxo3* was also dysregulated in bladder cancer and that the dysregulation of *circ-Foxo3* could potentially contribute to various solid tumors as a common pathogenic factor.

To date, the detailed mechanism by which *circ-Foxo3* regulates tumor progression has not be well understood. Several prior reports suggest the mechanism appears to be multifold. *circ-Foxo3* has been shown to increase cellular senescence,[14] to arrest cancer cell cycle progression by binding to the cell cycle proteins CDK2 and p21, and to inhibit angiogenesis.[25] Our data showed in bladder cancer tissue and

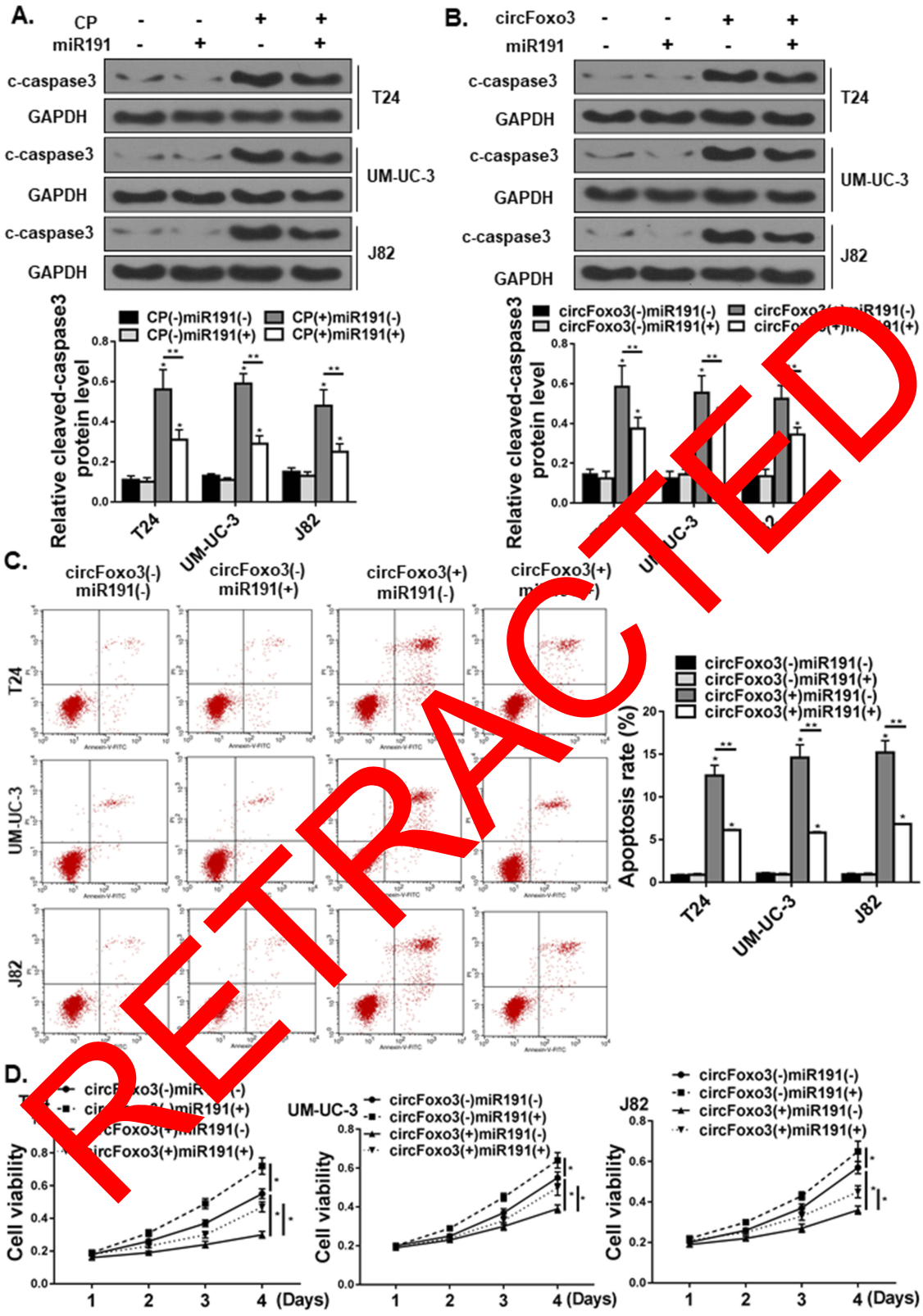

Figure 6 *miR-191-5p* suppressed the pro-apoptotic effect of *circ-Foxo3* in bladder cancer cells. (**A**) T24, UM-UC-3, and J82 bladder cancer cell lines were transfected with *miR-191* mimic (10 nM) and treated with cisplatin (CP, 2 μg/mL). The expression of cleaved-caspase3 (c-caspase3) was examined by Western blot and quantified relative to GAPDH (n=5). (**B**–**C**) T24, UM-UC-3, and J82 bladder cancer cell lines were co-transfected with *miR-191* mimic and a *circ-Foxo3* over-expression plasmid (n=5). The expression of cleaved-caspase3 (c-caspase3) was examined by Western blot (B) and apoptosis was analyzed by flow cytometry (**C**). (**D**) Cell viability measured by the CCK-8 assay in T24, UM-UC-3, and J82 bladder cancer cell lines receiving *miR-191* mimic or *circ-Foxo3* transfection (n=5). Mean ± SD, **p*<0.05, ***p*<0.01.

bladder cancer cell lines, one major effect of *circ-Foxo3* was the induction of apoptosis, indicated by increased cleaved-caspase3 and Bax/Bcl2 ratio. Our results was consistent with the pro-apoptotic effect of *circ-Foxo3* in breast carcinoma biopsies and in multiple cancer cell lines.[13] Importantly, in breast cancer cells, *circ-Foxo3* also increased Foxo3 protein level and promoted p53 ubiquitination and subsequent degradation, suggesting an alternative pathway by which *circ-Foxo3* induces cancer cell apoptosis.[13]

The role of non-coding circRNAs as potential microRNA "sponges" have been recognized.[8,27–31] Compared with most linear RNAs, circRNAs have no 3′ ends and are therefore more resistant to exonuclease degradation.[8] In addition, circRNAs are characterized by high sponging capacity (sequence containing multiple microRNA binding sites) and relatively high expression level. All these characteristics indicate circRNAs may be more effective microRNA-sponges than the linear non-coding RNAs. The sponging effect of circRNAs appears to be microRNA- and tissue-specific: certain circRNAs only "sponge up" microRNAs of a particular family in particular tissues.[32] However, the exact microRNA-sponging spectrum of *circ-Foxo3* has been controversial. For instance, in non-small cell lung cancer, *circ-Foxo3* sequesters miR-155;[12] in breast cancer, *circ-Foxo3* sequesters eight miRNAs.[25] We found for the first time that in bladder cancer cells, there was a strong negative correlation between the expression of *circ-Foxo3* and *miR-191*. Our experiments confirmed the 3′-UTR of *circ-Foxo3* was a direct binding site for *miR-191*, and the pro-apoptotic effect of *circ-Foxo3* could be effectively blocked by *miR-191* mimic. These results highlighted a critical role of the direct interaction between *circ-Foxo3* and *miR-191* in regulating bladder cancer apoptosis. Given that the aberrant expression of microRNAs in cancers are highly tissue-specific, this particular interaction between *circ-Foxo3* and *miR-191* may be indicative of a highly specific therapeutic target for bladder cancer.[33,34]

Our study has several limitations. First, there may be alternative pathways by which *circ-Foxo3* promotes cancer apoptosis,[13] which were not examined in our study. In addition, our focus of bladder-cancer-related microRNA is limited to *miR-191* and non-exhaustive. There could potentially be many other functionally important microRNAs that interact with *circ-Foxo3* in bladder cancer cells.

Conclusion

circ-Foxo3 regulated bladder cancer growth in vivo and in vitro. Overexpression of *circ-Foxo3* promoted bladder cancer apoptosis, which was partially attributable to the direct targeting of *circ-Foxo3* by *miR-191*.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Harbin Medical University. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki. All patients and healthy volunteers provided written informed consent prior to their inclusion within the study.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Abbreviations

circRNA, circular RNA; 3′-UTR, 3′-untranslated region; ANOVA, analysis of variance; BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; PBS, phosphate-buffered saline; RT-qPCR, Reverse transcription-quantitative polymerase chain reaction; SD, standard deviation; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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Disclosure

The authors declare that they have no competing interests in this work.

References

1. Antoni S, Ferlay J, Soerjomataram I, Znaor A, Jemal A, Bray F. Bladder cancer incidence and mortality: a global overview and recent trends. *Eur Urol*. 2017;71(1):96–108. doi:10.1016/j.eururo.2016.06.010
2. Pal SK, Miller MJ, Agarwal N, et al. Clinical cancer advances 2019: annual report on progress against cancer from the American society of clinical oncology. *J Clin Oncol*. 2019;37(10):834–849. doi:10.1200/JCO.18.02037
3. Danan M, Schwartz S, Edelheit S, Sorek R. Transcriptome-wide discovery of circular RNAs in archaea. *Nucleic Acids Res*. 2012;40(7):3131–3142. doi:10.1093/nar/gkr1009
4. Pamudurti NR, Bartok O, Jens M, et al. Translation of CircRNAs. *Mol Cell*. 2017;66(1):9–21 e27. doi:10.1016/j.molcel.2017.02.021
5. Granados-Riveron JT, Aquino-Jarquin G. The complexity of the translation ability of circRNAs. *Biochim Biophys Acta*. 2016;1859(10):1245–1251. doi:10.1016/j.bbagrm.2016.07.009

6. Memczak S, Jens M, Elefsinioti A, et al. Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature*. 2013;495 (7441):333–338. doi:10.1038/nature11928
7. Tang Z, Li X, Zhao J, et al. TRCirc: a resource for transcriptional regulation information of circRNAs. *Brief Bioinform*. 2018. doi:10.1093/bib/bby083
8. Wilusz JE, Sharp PA. Molecular biology. A circuitous route to noncoding RNA. *Science*. 2013;340(6131):440–441. doi:10.1126/science.1238522
9. Anderson MJ, Viars CS, Czekay S, Cavenee WK, Arden KC. Cloning and characterization of three human forkhead genes that comprise an FKHR-like gene subfamily. *Genomics*. 1998;47 (2):187–199. doi:10.1006/geno.1997.5122
10. Du WW, Yang W, Liu E, Yang Z, Dhaliwal P, Yang BB. Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2. *Nucleic Acids Res*. 2016;44 (6):2846–2858. doi:10.1093/nar/gkw027
11. Lu WY. Roles of the circular RNA *circ-Foxo3* in breast cancer progression. *Cell Cycle*. 2017;16(7):589–590. doi:10.1080/ 15384101.2017.1278935
12. Zhang Y, Zhao H, Zhang L. Identification of the tumorsuppressive function of circular RNA FOXO3 in nonsmall cell lung cancer through sponging miR155. *Mol Med Rep*. 2018;17(6):7692–7700. doi:10.3892/mmr.2018.8830
13. Du WW, Fang L, Yang W, et al. Induction of tumor apoptosis through a circular RNA enhancing Foxo3 activity. *Cell Death Differ*. 2017;24 (2):357–370. doi:10.1038/cdd.2016.133
14. Du WW, Yang W, Chen Y, et al. Foxo3 circular RNA promotes cardiac senescence by modulating multiple factors associated with stress and senescence responses. *Eur Heart J*. 2017;38(18):1402–1412. doi:10.1093/eurheartj/ehw001
15. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 2009;55(4):611–622. doi:10.13 clinchem.2008.112797
16. Wang L, Hou Y, Yin X, et al. Rottlerin inhibits cell growth and invasion via down-regulation of Cdc20 in glioma cells. *Oncotarget*. 2016;7(43):69770–69782. doi:10.18632/oncotarget.11 4
17. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identificat n of prog mmed cell death in situ via specific labeling of nuclear D A fragm *J Cell Biol*. 1992;119(3):493–501. doi:10.1083 .119 9
18. Nagpal N, Ahmad HM, Chameettacha S, Sundar Ghosh S, Kulshreshtha R. HIF-inducible miR-1 p motes migrati in breast cancer through complex regulation of TGFβ -signaling in ypoxic microenvironment. *Sci Rep*. 201 5:9650. doi: 038/srep09650
19. Liu Z, Huang S. Inhibition of miR-191 contri s to radiation-resistance of two lung ca cell line by altering autophagy activity. *Cancer Cell Int*. 2015;):16. d 10.1186/s12935-015-0165-5
20. Song Z, Ren H, Gao S, X, Zhang , Hao J. The clinical significance and g tion m anism hypoxia-inducible factor-1 and miR- 1 exp sion in atic cancer. *Tumour Biol*. 2014;35(11319–11 28. doi:10.1 07/s13277-014-2452-5
21. Di Leva G, Piovan C, Gasparini P, et al. Estrogen mediated-activation of miR-191/425 cluster modulates tumorigenicity of breast cancer cells depending on estrogen receptor status. *PLoS Genet*. 2013;9(3): e1003311. doi:10.1371/journal.pgen.1003311
22. Patnaik SK, Kannisto E, Yendamuri S, Blagosklonny MV. Overexpression of microRNA miR-30a or miR-191 in A549 lung cancer or BEAS-2B normal lung cell lines does not alter phenotype. *PLoS One*. 2010;5(2):e9219. doi:10.1371/journal.pone.0009219
23. Shen J, DiCioccio R, Odunsi K, Lele SB, Zhao H. Novel genetic variants in miR-191 gene and familial ovarian cancer. *BMC Cancer*. 2010;10:47. doi:10.1186/1471-2407-10-663
24. Chen P, Pan X, Zhao L, et al. MicroRNA-191-5p exerts a tumor suppressive role in renal cell carcinoma. *Exp Ther Med*. 2018;15 (2):1686–1693. doi:10.3892/etm.2017.5581
25. Yang W, Du WW, Li X, Yee AJ, Yang BB ctivity promoted by non-coding effects of circular RNA d Foxo3 p dogene in the inhibition of tumor growth and an genesis. *Onco e*. 2016;35 (30):3919–3931. doi:10.1038/on 2015 0
26. Lu J, Zhang PY, Li P, et al. rcular RN hsa_circ 001368 suppresses the progression of tric cancer by re lati miR-6506-5p/ FOXO3 axis. *Biochem ophys R Commun*. 019;512(1):29–33. doi:10.1016/j.bbrc.2019.02 11
27. Su H, Tao T, Yan z, et al. C ular RNA FRC acts as the sponge of MicroRNA to promote dde carcinoma progression. *Mol Cancer*. 201 18(1) 7. doi:10.1186 2943-019-1010-6
28. Han D, Li J, Wang t al. Circular RNA circMTO1 acts as the spon microRNA-9 suppress hepatocellular carcinoma progr sion. *Hepatology*. 2017; 6(4):1151–1164. doi:10.1002/hep.29270
29. o J, Cheng X Zhu H, Wang L, Liu L. Circular RNA-0007874 (c cMTO1) reve s chemoresistance to temozolomide by acting as a sp ge of micr NA-630 in glioblastoma. *Cell Biol Int*. 2018. doi: 002/ 11080
30. Wang J, Zhao SY, Ouyang SS, Huang ZK, Luo Q, Liao L. [Circular circHIPK3 acts as the sponge of microRNA-124 to promote human oral squamous cell carcinoma cells proliferation]. *Zhonghua Kou Qiang Yi Xue Za Zhi*. 2018;53(8):546–551. doi:10.3760/cma.j.issn.1002-0098.2018.08.009
31. Zhang X, Wang S, Wang H, et al. Circular RNA circNRIP1 acts as a microRNA-149-5p sponge to promote gastric cancer progression via the AKT1/mTOR pathway. *Mol Cancer*. 2019;18(1):20. doi:10.1186/ s12943-019-1010-6
32. Ebert MS, Sharp PA. MicroRNA sponges: progress and possibilities. *RNA*. 2010;16(11):2043–2050. doi:10.1261/rna.2414110
33. Yoshino H, Seki N, Itesako T, Chiyomaru T, Nakagawa M, Enokida H. Aberrant expression of microRNAs in bladder cancer. *Nat Rev Urol*. 2013;10(7):396–404. doi:10.1038/nrurol.2013.113
34. Amuran GG, Eyuboglu IP, Tinay I, Akkiprik M. New insights in bladder cancer diagnosis: urinary miRNAs and proteins. *Med Sci*. 2018;6(4):113.