ORIGINAL RESEARCH

RETRACTED ARTICLE: Circular RNA *circ-Foxo3* induced cell apoptosis in urothelial carcinoma via interaction with *miR-191-5p*

This article was published in the following Dove Press journal: OncoTargets and Therapy

Chunyang Wang¹ Weiyang Tao² Shaobin Ni¹ Qiyin Chen¹

¹Department of Urology, The First Affiliated Hospital of Harbin Medical University, Harbin City, Heilongjiang Province 150001, People's Republic of China; ²Department of Breast Surgery, The Third Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang Province 150001, People's Republic of China **Background:** Circular RNAs (circRNAs) play a critical region cancer. Energing evidence has shown circ-Foxo3, a circRNA, was dysregulated if a variation of tumor opes. However, the exact role of *circ-Foxo3* in bladder cancer has over been studied.

Methods: We measured the expression level or circ-Fords in human and murine bladder cancer tissues and in various human bladder cancer before. We aduced bladder cancer in mice by a carcinogen N-butyl-N-(4-hyder ybutyl)nitre miner aBN). circ-Foxo3 was over-expressed in mice by lentiviral gape transfer and in the order cells via overexpression plasmid. The effect of circ-Foxo3 on apoptosis has examined via apoptotic marker staining, Western blot, and flow cytor cary. We further cancer circe the interaction between circ-Foxo3 and miR-191 and its functional impact on bladder cancer cells.

Results: circ-Foxo3 was a wnregulated in bladder cancer in vivo and in vitro, and was upregulated in response to approach of circ-Foxo3 promoted bladder cancer cell apoptos in BBN included in human bladder cancer cell lines. miR-191-5p suppressed circ-Foxo3 evolution and the pro-apoptotic effect of *circ-Foxo3* in bladder cancer cells via dire the cargeting the 3'-untranslated region (3'-UTR) of *circ-Foxo3*.

Correction circ-For b3 was downregulated in bladder cancer in vivo and in vitro, and premoted blader cancer apoptosis via direct interaction with miR-191. circ-Foxo3 could be a premit therapeut target for bladder cancer.

Keywerds: 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 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

OncoTargets and Therapy 2019:12 8085–8094

OncoTargets and Ther

Correspondence: Chunyang Wang Department of Urology, The First Affiliated Hospital of Harbin Medical University, No.23 Youzheng Street, Harbin, Heilongjiang Province 150001, People's Republic of China Tel +86 I 524 511 0305 Email szlvyih446620192@126.com



COUSS CO

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-191circ-Foxo3* axis appeared critical to bladder cancer apoptosis.

Methods

Human bladder cancer samples

The study involving human samples was approved by t Institutional Review Board of The First Affiliated Hospital of Harbin Medical University. Written inform *.*a sent was obtained from all patients upon recruiting. Thirt pairs of fresh bladder cancer tissues and adjecent portal blac der tissue were excised during part a or radie cystectomy from patients with confirmer demosis of unbelial carcinoma at the Department of Urok, of The First Affiliated Hospital of Harm Medical University between 2016 and 2017. None of the plants had medication or radiation therapy prior rgery. The specimens were snap-frozen in June itroge impediately after excision. Pathological and hist ogical dis noses were performed by two pathology is independently, and the diagnosis of urothelial carcine has a was confirmed in all samples. The grades and stages of pecimens 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% CO₂. 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 (H₂O₂), or 1 μ g/mL doxorubicin (DOX), or 2 μ g/mL cisplatin (CP) for 24 h.

Bladder tumor model in mice

All animal protocols were approved by the Ethical Committee of The First Affiliat Hospit of Harbin Medical University and adheren to the Gui for the Care and Use of Laborate Animals (NRC 2011, 8th Edition). Male, 8-week- a C57PL/6 n. . ere obtained from The First Affilied Hopital of Harbin Medical University. Mice ere housed in a temperature-controlled environment 12-h light dy ycles and unrestricted access to food and inking water. To induce carcinogenesis in _____e bladder, 25% N-butyl-N-(4-hydroxybutyl) amine (BBN; Tokyo Chemical Industry Co Ltd, nitre Jap) was add to the drinking water for 17 weeks ssue har sting. BBN-free water (vehicle) was until drinking water in equal volume for added h. as control treatment. The induction of bladder 17 ancer was histologically confirmed at tissue harvesting.

listology

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 $(2 \times 10^8$ viral genome copies/µL) was injected into the male 8week-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 μ g total RNA input in 20 μ L reaction. A portion of the reverse transcription product (1 μ L, equal to 0.1 μ g cDNA) was saved for regular PCR with two appropriate primers. qPCR was performed using the miScript SYBR Green PCR Kit (Qiagen, USA) the 1 μ L cDNA template. The relative expression of NA was quantified using the $\Delta\Delta$ Cq method.¹⁵

Apoptosis assay

ates a transfection. Cells were grown on 6-well Annexin V NTC apop-Apoptosis was quantified usiz tosis kit (Thermo Fisher Scientific, UN). Briefly, cells were detached 48 h post-trappection, washed h PBS, and resuspended in 500 µL rding by er containing 5 µL propidium iodide (PI) and 5 µL v xin V-FTC. After incubation at for It nin, cost were washed and resusroom tempe pend in oinding buffer. Doptosis was analyzed by a FACSca. ur f er (BD Biosciences, USA).

Western bit

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-polyacry-lamide 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 μ L/well medium for 24 h. CCK-8 solution was added to the plates (10 μ L/well) at 0 h, 24 h, 48 h, 72 h, and 96 h, respectively, and for the plated for 4 hrs for colorimetric reaction. Absc bance at 4.0 nm was measured with a microplate reade to represent ell viability.

Luciferase reporter asay

The 3'-untransland resord -UTR) of circ-Foxo3 RNA containing the prative miR-N binding sequence was amplified by PCR do ned into a GLO (Promega, USA) dualluciferase miRNA rget expression vector (named pmirGlo-**MAT**; WT). **Network** was genrated within the circ-Foxo3 3'-UTR sequence by a Iulti Site-Directed Mutagenesis Kit (Agilent uikChange Tempologie (USA) resulting a mutant luciferase reporter vector (named pmirGlo-CREB1-MUT; MUT). For the luciter. 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 posttransfection 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 \pm 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 the apoptotic protein markers was markedly increased following these treatments, as indicated by increased cleaved-caspase3 expression and increased ratio of the pro-apoptotic protein Bax over the pro-survival protein Bcl2 (Figure 2A).

We further characterized the apoptotic stress in additional cell lines. The expression of *circ-lineo3* was significantly upregulated in bladder cancer centines 24, UM-UC3 and J82 as well as the normal hadder epitheral cell line SV-HUC-1 upon treatment and doxerubicin (Figure 2B), cisplatin (Figure 2C), and O_2 (Figure 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, Wertan or eshowed that the expression the cleaved-caspace and the rate of Bax to Bcl2 was increased in mice received *circ-Foxo3* lentiviral injection (Figure 3B).

We then examined are effect of *circ-Fo.03* overexpression on apoptosis to via the transitivity overexpressed *circ-Foxo3* RN4 in human and der tancer cell lines with a *circ-Foxo3* clasmic (Figure 4A). Overexpression of *circ-Foxo3* promoted blacter cancer cell apoptosis, as measured by Western blot of apoptotic markers (Figure 4B) and low cytometry (Figure 4C).

In thermore, we examined the viability of bladder cancer all lines. Overexpression of *circ-Foxo3* signifiearly reduced bladder cancer cell viability as measured by the C-K-8 assay (Figure 4D).

niR-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 2 *circ-Foxo3* was upregulated in response to apoptotic stress. (**A**) Western blot of cleave caspases p caspase3), Bax Bcl2 and GAPDH expression in J82 cells treated with vehicle (Ctrl), doxorubicin (Dox; μ g/mL), cisplatin (CP; 2 μ g/mL), and H₂O₂ (H2O2; 0.4 mM). The set Lintensity 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 bladde treated with vehicle (**B**–**D**), doxorubicin (**B**), cisplatin (**C**), and H₂O₂ (**D**), respectively (n=5). Mean ± SD, *p<0.05.



Figure 3 *circ-Foxo*3 proted bladder tumor cell apoptosis in BBN mice. Male, 8-week-old C57BL/6 mice received control (circControl-GFP) and *circ-Foxo*3 (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 \pm 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 (<u>http://www.targetscan.org</u>) (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-fox induced to the produced viability in bladder cancer cells. T24, UM-UC-3, and J82 bladder cancer cell lines received control (Ctrl) or circ-foxo3 overexpression (circ-foxo) blands. (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 =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 Foxo3 and miR NA expression in tumor biopsy tissue /ww.ta ed a putative *miR-191-5p* target site in the from bladder cancer patients (n=30). (B) Bioinformatic analysis using the TargetScan algorithm (http scan.org/) pred 3'-UTR of circ-Foxo3. (C) Bladder cell lines were transfected with a non-targeting microRNA (Ctrl), or a miR-19 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 vector carrying the wild-type (pmirGlo-CREBI-WT; uciferase report WT) or mutant (pmirGlo-CREBI-MUT; MUT) 3'-UTR sequence of circ-Foxo3 were transf ed into J82 cells in combine on with a miR-191-5p mimic (miR-191-5p) or a nonalized to Renilla Inciferase activity) was analyzed 48 hrs post-transfection. (**E**) The targeting microRNA mimic (Control). The relative luciferase activity (arbitrary units, no expression of circ-Foxo3 was measured by RT-qPCR in bladder cancer cells receiving nor rgeting microRN (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 suppressed by *miR-191* in bladder cancer cells.

miR-191-5p suppressed approtosization inhibiting circ-Foxo3 in pladde cancer cells

To elucidate the functional relevant of the interaction *circ-Foxo3*, we asfected bladder between miR-191 and cancer cells with *miR-1-5p* mimic, and treated cells with cisplatin to inc apoptosi Western blot showed cisplatin-ip acea popto. way significantly attenuated by ession o overex miR-191 (rigure 6A). Notably, overex*c-Foxo-* alone also increased apoptosis pression (Figure 6B). -transfection with *miR-191* mimic, however, effectively suppressed 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 prosurvival 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 μ g/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 cleavedcaspase3 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 microRNAsponges than the linear non-coding RNAs. The sponging effect of circRNAs appears to be microRNA- and tissuespecific: 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 sequesters eight miRNAs.²⁵ We found for the first time that in bladder cancer cells, there was a strong neartive con tion between the expression of *circ-Foxo*² and m -191was was Our experiments confirmed the 3'-UTK of *circ-V* direct binding site for miR-191, ap the p. optotic effect of circ-Foxo3 could be effected by blocked by miR-191 mimic. These results highlighted a titical role of the direct interaction between *circoxo3* and *m*191 in regulating bladder cancer apopters. Given that the aberrant expression of microRNAs in uncertaire highly tissue-specific, this tion by yeen cire coxo3 and miR-191 may particular inter be indicat e of a view therapeutic target for bladighly s der care r.^{33,34}

Our sub-has several limitations. First, there may be alternative paravays 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 prominent study.

Availability of taken d marerials

The datasets used an or analysed a singule current study are available from the corresponding author on reasonable request.

Abbreviations

circuit, circular VIA; 3'-UTR, 3'-untranslated region; NOVA, analysis of variance; BBN, N-butyl-N-(4-hydrovbutyl)nitro mine; PBS, phosphate-buffered saline; RTq, SR, Revere transcription-quantitative polymerase chain reaction, 5D, standard deviation; TUNEL, terminal deoxytempotidyl transferase dUTP nick end labeling.

Acknowledgment

This study was supported by the postdoctoral scientific research developmental fund of Heilongjiang Province (Grant No. LBH-Q17103)

Disclosure

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

References

- 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
- 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
- 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
- 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
- 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 an invasion via down-regulation of Cdc20 in glioma cells otarget 2016;7(43):69770-69782. doi:10.18632/oncotarget.12
- f of prog 17. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identificat hmed cell death in situ via specific labeling of nuclear fragm Cell Biol. 1992;119(3):493-501. doi:10.1083 0.119
- 18. Nagpal N, Ahmad HM, Chameettachar S, Sundar Ghosh S. Kulshreshtha R. HIF-inducible miR-12 notes migration n breast cancer through complex regulation TGF signaling in ypoxic microenvironment. Sci Rep. 2015:9650. doi: 038/srep09650
- 19. Liu Z, Huang S. Inhibition of miR-191 contrib s to radiationresistance of two lung carror cell line by altering autophagy activity. *Cancer Cell Int.* 2015;1: 0):16. doi:10.1186/s12935-015-0165-5 Song Z, Ren H, Gao S, Jone X, Zhang J, Hao J. The clinical significance and constraint management on hypoxia-inducible factor-
- 20. Song Z, Ren H, Gao S, nism any nypoxia-inducible factor-presentic cancer. *Tumour Biol*. 1 expi 1 and miR sion in 11319-11 28. doi:10.1. 37/s13277-014-2452-5 2014;35(1

- 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 a Foxo3 p. by non-coding effects of circular RNA dogene in the inhibition of tumor growth and an genesis. Onco, ne. 2016:35 (30):3919-3931. doi:10.1038/one 2015.
- 26. Lu J, Zhang PY, Li P, et al. cular RN hsa_circ 01368 suppresses the progression of tric cancer by it lati _miR-6506-5p/ FOXO3 axis. Biochem ophys R Commun. J19;512(1):29–33. doi:10.1016/j.bbrc.2019.02
- z, et al. ılar RNA FRC acts as the sponge 27. Su H, Tao T, Yap to promote der arcinoma progression. Mol of MicroRNA Cancer. 201,18(1) 7. doi:10.1186 2943-019-1010-6
- 28. Han D, Li J, Wang et al. Circular RNA circMTO1 acts as the nicroRNA-9 suppress hepatocellular carcinoma prospop 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 cMTO1) revei s chemoresistance to temozolomide by acting as a ge of micr NA-630 in glioblastoma. Cell Biol Int. 2018. st 002/01 .11080 doi:
 - Wang J, Znao SY, Ouyang SS, Huang ZK, Luo Q, Liao L. [Circular ircHIPK3 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
 - 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.

OncoTargets and Therapy

Publish your work in this journal

OncoTargets and Therapy is an international, peer-reviewed, open access journal focusing on the pathological basis of all cancers, potential targets for therapy and treatment protocols employed to improve the management of cancer patients. The journal also focuses on the impact of management programs and new therapeutic

Submit your manuscript here: https://www.dovepress.com/oncotargets-and-therapy-journal

Dovepress

agents and protocols on patient perspectives such as quality of life, adherence and satisfaction. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit http://www.dovepress.com/ testimonials.php to read real quotes from published authors.