Circ-FOXO3 inhibits triple-negative breast cancer progression via interaction with WHSC1

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Abstract

Background. Circular RNAs (circRNAs), a subclass of non-coding RNAs characterized by covalently closed continuous loops, play a key role in tumorigenesis and aggressiveness. However, the potential molecular mechanism of circRNAs in triple-negative breast cancer (TNBC) remains largely unknown. Exploring their roles and mechanisms in TNBC progression may help identify new diagnostic markers and therapeutic targets.

Methods. Differentially expressed circRNAs were identified by RNA sequencing. The expression of circ-FOXO3 was evaluated using Real-time quantitative RT-PCR and RNA in situ hybridization. The impacts of circ-FOXO3 on the TNBC progression were investigated by in vitro and in vivo functional assays. The underlying mechanism of circ-FOXO3 was revealed by Western blot, RNA pull down, mass spectrometry, RNA immunoprecipitation, luciferase assays and rescue experiments.

Results. Here, we identified circ-FOXO3 as a tumor suppressor in TNBC progression. Circ-FOXO3 was predominantly expressed in the cytoplasm and dramatically downregulated in TNBC tissues. Notably, circ-FOXO3 expression was significantly lower in blood samples from patients with TNBC. Low circ-FOXO3 expression in TNBC tissues and blood was associated with lymph node metastasis and unfavorable outcomes in patients with TNBC. In addition, circ-FOXO3 overexpression inhibited the growth, invasion, and metastasis of TNBC cells both in vitro and in vivo. Moreover, we demonstrated that circ-FOXO3 interacts with Wolf-Hirschhorn syndrome candidate 1 (WHSC1) and inhibits WHSC1 activity, resulting in the inhibition of H3K36me2 modifications at the Zeb2 promoter, ultimately inhibiting Zeb2 expression and halting cancer progression.

Conclusion. Taken together, these results reveal the tumor-suppressive functions of circ-FOXO3 in inhibiting WHSC1-mediated H3K36me2 modification of Zeb2, suggesting that circ-FOXO3 could serve as a potential novel predictive prognostic biomarker and therapeutic target for TNBC.

Background

Globally, breast cancer has become the malignancy with the highest morbidity and mortality rates among women. Despite significant advances in early detection and treatment, breast cancer remains a major problem affecting women's health [1]. Breast cancer can be divided into four subtypes based on molecular properties: luminal A, luminal B, HER2-positive, and triple-negative breast cancer (TNBC). TNBC, which lacks the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), accounts for 15–20% of all breast cancers and has the highest frequency of recurrence and metastasis [2]. Furthermore, patients with TNBC are usually diagnosed at a late stage and do not benefit from hormonal or targeted therapies because of the lack of biomarkers, which facilitate early diagnosis and precise treatment, leading to a poor prognosis [3]. Therefore, there is an urgent need to develop novel prognostic biomarkers and therapeutic targets for TNBC treatment.
Circular RNAs (circRNAs) are a class of endogenous single-stranded non-coding RNAs. It is a covalently bound circular RNA formed by reverse splicing. Unlike linear RNA, circRNA does not have a 5’ terminal cap and a 3’ terminal poly (A) tail, which renders them resistant to nucleic acid endonucleases [4]. Human cells are usually dominated by circRNAs composed of exons, generally consisting of 1–5 exons and 100 bp–3 kb in length, which are typically localized in the cytoplasm [5]. circRNAs can regulate gene transcription and protein activity as competitive endogenous RNA sponges or binding proteins [6]. Studies have confirmed that circRNAs are abundant in eukaryotes and play critical roles in various diseases, including cancer [7]. However, the role of circRNAs in TNBC progression and the underlying mechanisms warrant further investigation.

Histone modifications refer to the processes of methylation, acetylation, ubiquitination, and other modifications of histones under the action of relevant enzymes that tune gene expression by affecting chromatin architecture or the affinity of transcription factors to the promoters of structural genes [8]. Histone methylation is regulated by a combination of methyltransferases and demethylases. Aberrant histone methylation, mediated by abnormal enzyme expression or activity, is closely associated with tumor development and metastasis [9, 10].

In this study, we analyzed the expression profiles of circRNAs in TNBC and found that circ-FOXO3 in TNBC tissues was significantly lower in TNBC tissues than that in non-TNBC and normal tissues. circ-FOXO3 overexpression suppressed the proliferation, invasion, and metastasis of TNBC cells both in vitro and in vivo. We further identified the histone 3 lysine 36 (H3K36)-specific methyltransferase WHSC1 as a protein partner of circ-FOXO3. Circ-FOXO3 binds to WHSC1 and inhibits its cytoplasmic localization and activity, which in turn suppresses H3K36me2 modification of the Zeb2 promoter and Zeb2 expression, ultimately inhibiting TNBC progression. In addition, low blood circ-FOXO3 expression was detected in patients with TBNC and was associated with TNBC-related metastasis.

**Methods**

**Cell culture**

The normal breast mammary epithelial cell line MCF-10A, and human breast cancer cell line MCF-7, T47D, ZR75-1, MDA-MB-231, BT549, BT474, SKBR3, HCC1937 and HCC38 were obtained from the American Type Culture Collection. Cell lines were reauthenticated by Short Tandem Repeat (STR) analysis every 6 months after resuscitation in our laboratory. MCF-10A cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, Logan, UT, USA) supplemented with 5% horse serum, 20 μg/mL EGF, 0.5 μg/mL hydrocortisone, 0.1 μg/mL cholera toxin, 10 μg/mL insulin, and 1% penicillin/streptomycin. Breast cancer cells were cultured in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator of 5% CO₂ at 37 °C.

**Patients and Specimens**
Fifty-four fresh primary tumor tissues and eighteen adjacent normal tissues (more than 5 cm away from the tumor margin) were obtained from patients diagnosed with breast cancer who underwent complete resection in the Affiliated Tumor Hospital of Guangzhou Medical University. Three matched pairs of TNBC tissues were used for high-throughput sequencing. Primary tumor specimens were obtained from fifty-one patients diagnosed with TNBC who underwent complete resection in the Affiliated Cancer Hospital of Guangzhou Medical University between 2004 and 2008. Follow-up information was obtained from review of the patients’ medical record. This study was approved by the Ethics Committee of Affiliated Cancer Hospital of Guangzhou Medical University and written informed consent was provided by all patients based on the Declaration of Helsinki.

**Plasmids, shRNAs and stable cell lines construction**

The sequence of circ-FOXO3 was amplified and cloned into a circRNA overexpression vector pLCDH-ciR (Geneseed, Guangzhou, China). Through restriction enzyme sites EcoRI and BamHI, and also confirmed by sequencing. Short hairpin sequences against WHSC1 or the scrambled short hairpin RNA (shRNA) sequences were cloned into the GFP-labeled lentiviral vector GV102 (GENECHEM). The target sequences selected are: shControl, TTCTCCGAACGTGTCACGT; shWHSC1-1, CCTAAACCATGGTTCTAAA; shWHSC1-2, GGGCGATCCTAAGAAGAAA; shWHSC1-3, GAAGAGAGGTTCAGCTAAA. Expression plasmid for pCMV6-Zeb2, pCMV6-WHSC1 and pCMV6-XL5 empty plasmid were purchased from Origene (Rockville, MD). For cell transfection, Lipofectamine 2000 reagent (Invitrogen) was used according to the manufacturer’s instructions. After 72 h transfection, stably expressing or knockdown cells were selected in RPMI 1640 medium containing 1 μg/mL puromycin.

**Real-time quantitative RT-PCR**

Total RNA isolated from cells or tissues was used E.Z.N.A.® HP Total RNA Kit (Omega Bio-tek, Doraville, GA, USA). For circRNA detection, RNase R (3 U/mg, Epicenter) digestion was undertaken at 37°C for 15 minutes. Reverse transcription and real-time PCR were performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche), SYBR Green PCR Master Mix (Applied Biosystems). The results of transcript levels were analyzed by the $2^{-\Delta\Delta Ct}$ method. The primer sequences were shown in Supplementary Table S1.

**RNA sequence and data analysis**

High-throughput sequencing and subsequent bioinformatic analysis of circRNAs or mRNA were performed by Cloud-Seq Biotech (Shanghai, China). Total RNAs were treated using the Ribo-Zero rRNA Removal Kit (Illumina, CA, USA) to remove ribosomal RNA, and the resulting RNA was used to construct RNA libraries using the TruSeq Stranded Total RNA Library Prep Kit (Illumina) according to the manufacturer’s instructions. Libraries were quality-controlled and quantified using the BioAnalyzer 2100 System (Agilent Technologies, CA, USA), then sequenced (150 cycles) on the HiSeq 6000 platform (Illumina) according to the manufacturer’s instructions. After 3’ adaptor-trimming and low quality reads removing by cutadapt software (v1.9.3). For circRNA sequencing, the high quality clean reads were
aligned to the reference genome or transcriptome using STAR software (v2.5.1b). circRNAs were identified using DCC software (v0.4.4) and annotated using the circBase and circ2Traits databases. Data on the normalized and expression differences of circRNAs were analyzed using EdgeR software (v3.16.5). For mRNA sequencing, the high quality clean reads were aligned to the reference genome (UCSC hg19) with hisat2 software (v2.0.4). Then, guided by the Ensembl gtf gene annotation file, cuffdiff software (part of cufflinks) was used to get the gene level FPKM as the expression profiles of mRNA, and fold change (FC) and P value were calculated based on FPKM, differentially expressed mRNA (FC > 2, P < 0.05) were identified.

Nuclear-cytoplasmic fractionation

Nuclear and cytoplasm of cells were separated by Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, United States) following the manufacturer’s instructions.

Fluorescence in situ hybridization (FISH)

Cy3-labeled circ-FOXO3 FISH probe was designed and synthesized by Guangzhou RiboBio (Guangzhou, China). FISH was conducted using Ribo Fluorescent in Situ Hybridization Kit (RiboBio). For cell FISH assay, cells were fixed with 4% (w/v) paraformaldehyde (PFA), permeated with 0.5% triton X-100 and hybridized with circ-FOXO3 probe at 37 °C overnight. For tissue FISH assay, TNBC tissues were fixed, permeated and hybridized with circ-FOXO3 probe. The hybridization buffer was then gradually eluted with 4× saline-sodium citrate (SSC), 2× SSC and 1× SSC. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). The images were acquired on a Leica SP5 confocal microscope (Leica Microsystems, Mannheim, Germany).

RNA immunoprecipitation assay

RNA immunoprecipitation (RIP) assay was performed using Magna RIP RNA Binding Protein Immunoprecipitation Kit (Millipore), using antibodies specific for WHSC1 or IgG. Coprecipitated RNAs were detected by real-time quantitative RT-PCR.

Biotin-labeled RNA pull-down and mass spectrometry analysis

The 5’-monophosphorylated linear or random probe of circ-FOXO3 was in vitro transcribed using Biotin RNA Labeling Mix (Roche), reaction mixture containing excessive guanosine monophosphate (7.5 mmol/L) and guanosine triphosphate (1.5 mmol/L), and T7 RNA polymerase. After incubation with guide oligonucleotide targeting circular junction the 5’-monophosphorylated linear probe was circularized using T4 RNA ligase I, treated with RNase R, and purified with RNeasy Mini Kit (Qiagen Inc.). The lysates of 2 × 10^7 cancer cells were incubated with 3 μg of biotin-labeled probe, and treated with 35 μL of Streptavidin C1 magnetic beads (Invitrogen) for 1 h. The retrieved protein was detected by Western blot or mass spectrometry analysis.

CCK-8 assays
2 × 10⁴ cells were seeded in 100 μl of complete culture media in 96-well plates for various time periods. Cell Counting Kit-8 assay (APExBio) was performed to measure cell viability according to manufacturer’s instructions.

**Colony formation**

1 × 10⁴ cells were seeded into six-well plates in triplicate, incubated for 10–12 days, fixed with methanol for 20 min, and stained with crystal violet. Colonies larger than 50 cells were counted.

**Cell cycle analysis**

Cells were washed with PBS, and fixed with 70% ethanol (-20°C ice-cold) for 1 h at 4 °C. Fixed cells were washed with PBS and incubated with RNAse A (0.1 mg/mL) for 30 min followed by incubation with propidium iodide (50 μg/mL) for 30 min at room temperature. Cell cycle analysis was performed by flow cytometry (BD FACSCanto II, BD Biosciences).

**Wound-healing assay**

Cells were seeded into six-well plates at 5×10⁵ cells per well and allowed to grow to 90-95% confluence. A linear scratch was created using a sterile pipette. The cells were washed with PBS, and cultured with serum-free medium. Images were taken using a Leisa microscope and the distance migrated was observed at 0, 24h and 48h after wounding.

**Transwell assay**

Cell invasion was evaluated by Matrigel-coated Transwell and Transwell inserts (BD Biosciences, San Diego, CA, USA). In total, 3 × 10⁴ cells suspended in 200 μL of serum-free medium were added to the upper chamber. The lower chambers were filled with the normal culture medium. The cells were incubated for 24 h at 37 °C. After incubation, the cells on the upper surface were removed, and the cells on the lower surface were fixed and stained with 0.1% crystal violet. The number of invaded cells was counted under a microscope in five predetermined fields for each membrane at ×200 magnification.

**Western blotting**

Cells were lysed by RIPA buffer (Beyotime Biotechnology, China). Total protein concentrations were detected by BCA Protein Assay Kit (Thermo Fisher, Waltham, MA, USA). Separated by 8-12% SDS-PAGE, the proteins were then transferred to PVDF membranes. Blocked with 5% skim milk, the membranes were incubated with primary antibodies overnight at 4 °C and then the secondary antibodies for 1h at room temperature. Bound antibodies were visualized by ECL reagents (Thermo Fisher). The primary antibodies included anti-b-Actin antibody (#3700, Cell Signaling Technology), anti-WHSC1 antibody (#ab75359, Abcam), anti-H3K36me2 antibody (#ab176921, Abcam), anti-Zeb2 antibody (#97885, Cell Signaling Technology), anti-E-Cadherin antibody (#3195, Cell Signaling Technology), anti-Vimentin antibody (#5741, Cell Signaling Technology).
**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) was performed by using EZ-Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (Millipore, Billerica, MA, USA) according to the manufacturer’s protocols. Briefly, cells were fixed with 1% formaldehyde and quenched in 125 mM glycine. Protein-DNA complexes were immunoprecipitated using anti-WHSC1 antibody, anti-H3K36me2 antibody, or control IgG (Abcam). The ChIP DNA was isolated and amplified by PCR. The primer sequences were shown in Supplementary Table S1.

**Luciferase reporter assay**

The Zeb2 promoter was inserted into the pGL3-basic vector expressing luciferase (Promega, Madison, WI, USA). All constructs were verified by sequencing. Cells were transiently transfected with indicated pGL3-Zeb2 or pGL3-mut-Zeb2 using Lipofectamine 3000 (Invitrogen). The Renilla luciferase was as internal control. Luciferase activity was measured 48 hours after transfection using the Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol.

**In vivo proliferation and metastasis assays**

Four-week-old female BALB/c nude mice were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). All animal work was performed in accordance with protocols approved by the Animal Experimentation Ethics Committee of Guangzhou medical University. To evaluate effect of circ-FOXO3 on tumor growth, 5×10^5 MDA-MB-231 cells transfected with pLCDH-circ-FOXO3 or control vector were subcutaneously into the nude mice (n = 4 per group). Tumor size and body weight were measured every 3 days. The tumor volume was calculated using the formula: \( V = \frac{1}{2} \times L \times W^2 \), and growth curves were plotted using average tumor volume within each experimental group at the set time points. At the end of treatment, the animals were sacrificed, and the tumors were removed and weighed for use in immunohistochemical (IHC) staining. To evaluate effect of circ-FOXO3 on tumor metastasis, 1×10^5 MDA-MB-231 cells transfected with pLCDH-circ-FOXO3 or control vector were injected into the tail vein of nude mice (N=4 per group). After 2 months, mice were sacrificed. Their lungs were fixed in 4% paraformaldehyde, paraffin-embedded and sliced. Lung sections were stained by hematoxylin and eosin (H&E) and imaged. The numbers of micrometastases in the lungs per tissue section in individual mice were determined from morphological observation of H&E-stained sections.

**Statistical analysis**

Statistical analyses were performed using Prism 8.0.1 (GraphPad Software, San Diego, CA, USA) and SPSS 25.0 (IBM, SPSS, Chicago, IL, USA). The differences of clinicopathological features between groups were analyzed using Fisher’s exact test. Other differences between groups were assessed for significance using student’s t test or ANOVA as appropriate. Overall survival was analyzed using the Kaplan–Meier method, and survival curves were compared using the log-rank test. No blinding was used. Sample sizes...
were chosen without pre-specified effect sizes. Data were shown as mean ± standard deviation (SD). Differences that were associated with P < 0.05 were considered statistically significant.

Results

Circ-FOXO3 expression is downregulated in TNBC tissues and cells

To identify essential circRNAs expressed in TNBC tissues, we analyzed the expression profiles of circRNAs in three matched pairs of TNBC and adjacent normal tissues using high-throughput sequencing. A total of 38 dysregulated circRNAs were identified in TNBC tissues, of which 31 were upregulated and 7 were downregulated (Figure 1A, Supplementary Table S2). Among them, circ-FOXO3 was one of the most significantly downregulated circRNAs (with an average fold-change of −4.87, P < 0.01) in TNBC tissues (Figure 1A, Supplementary Table S2). Circ-FOXO3 is generated from exon 3 of FOXO3a and has a length of 1435 nt. The back-splice junction site of circ-FOXO3 was amplified using divergent primers and confirmed using Sanger sequencing (Figure 1B). RT-qPCR results showed that circ-FOXO3 had stronger resistance to RNase R than the linear form of FOXO3a in MDA-MB-231 cells (Figure 1C). RNA fluorescence in situ hybridization (RNA-FISH) and subcellular fractionation assays revealed abundant signals and enrichment of circ-FOXO3 in the cytoplasm (Figure 1D, Supplementary Figure S1). We further validated circ-FOXO3 expression in TNBC and adjacent normal tissues using RT-qPCR and confirmed that circ-FOXO3 expression was significantly downregulated in TNBC tissues compared to that in adjacent normal tissues (Figure 1E). Notably, circ-FOXO3 expression was significantly lower in TNBC tissues than in non-TNBC tissues (Figure 1F). In addition, circ-FOXO3 levels were significantly decreased in the TNBC cell lines (Figure 1G). Next, we analyzed the correlation between circ-FOXO3 expression and the clinicopathological features of patients with TNBC. Fluorescence in situ hybridization (FISH) using a biotin-labeled specific probe was further conducted to detect circ-FOXO3 expression in TNBC tissues (Figure 1H). We found that high circ-FOXO3 expression was negatively associated with lymph node metastasis in patients with TNBC (Supplementary Table S3). Finally, we investigated the correlation between circ-FOXO3 expression and the prognosis of patients with TNBC. We found that patients with TNBC with higher circ-FOXO3 expression showed a better prognosis according to the Kaplan–Meier survival analysis (Figure 1I). Together, our results indicate that circ-FOXO3 is frequently downregulated in TNBC and is inversely correlated with malignant features and poor prognosis of TNBC.

Circ-FOXO3 inhibits the proliferation and metastasis of TNBC cells

Next, we explored the biological role of circ-FOXO3 in TNBC progression. We constructed a circ-FOXO3 overexpression plasmid (Figure 2A) and confirmed that circ-FOXO3 was specifically and efficiently overexpressed in TNBC cells (Figure 2B). We performed colony formation and CCK-8 assays to assess the effect of circ-FOXO3 on cell proliferation. Circ-FOXO3 overexpression significantly inhibited colony formation (Figure 2C-D) and proliferation of TNBC cells (Figure 2E, Supplementary Figure S2A). Subsequently, cell cycle analysis showed that circ-FOXO3 overexpression decreased TNBC cell proliferation by triggering G2 phase arrest (Figure 2F, Supplementary Figure S2B). Moreover, in vivo
experiments showed that circ-FOXO3 overexpression markedly inhibited the tumor growth of TNBC cells (Figure 2G-I). IHC results revealed that the expression of the cell proliferation marker Ki67 was significantly decreased in tumors from the circ-FOXO3 overexpression group (Figure 2J). We examined the effects of circ-FOXO3 overexpression on TNBC cell migration and invasion. Wound healing and transwell assays showed that circ-FOXO3 overexpression significantly suppressed the migration and invasion of TNBC cells (Figure 3A-C). Since epithelial-mesenchymal transition (EMT) is pivotal for the metastatic potential of tumor cells[11], the protein levels of EMT markers were further examined. We found that circ-FOXO3 overexpression significantly upregulated the expression of the epithelial marker E-cadherin and decreased that of vimentin, a mesenchymal marker (Figure 3D), suggesting that circ-FOXO3 overexpression contributes to inhibiting the EMT in TNBC cells. In the experimental metastasis assay, nude mice treated with tail vein injections of MDA-MB-231 cells stably transfected with circ-FOXO3 displayed fewer lung metastatic colonies (Figure 3E-G), suggesting that circ-FOXO3 overexpression markedly inhibited TNBC cell metastasis. Altogether, these results indicated that circ-FOXO3 suppressed the proliferation and metastasis of TNBC cells both in vivo and in vitro.

Circ-FOXO3 suppressed TNBC cell proliferation and metastasis via Zeb2 inhibition

To elucidate the mechanisms underlying the tumor-suppressive roles of circ-FOXO3, we analyzed differentially expressed genes after circ-FOXO3 overexpression using RNA sequencing. RNA-seq revealed that 2212 transcripts were significantly upregulated (fold-change > 2), while 2083 transcripts were significantly downregulated (fold-change < 0.5) in circ-FOXO3-overexpressing MDA-MB-231 cells (Figure 4A). Intriguingly, the mRNA expression of the EMT inducer Zeb2 was significantly decreased in circ-FOXO3-overexpressing MDA-MB-231 cells compared to control cells (Figure 4B-C). qRT-PCR and western blotting further confirmed that circ-FOXO3 significantly decreased Zeb2 expression in TNBC cells (Figure 4D-E). To further investigate the role of Zeb2 in circ-FOXO3-mediated inhibition of TNBC cell proliferation and metastasis, we rescued Zeb2 expression in circ-FOXO3-overexpressing TNBC cells (Figure 4F and Supplementary Figure S3A). CCK8 and colony formation assays revealed that Zeb2 overexpression prevented circ-FOXO3-mediated inhibition of TNBC cell proliferation (Figure 4G-H, Supplementary Figure S3B). Furthermore, wound healing and transwell assays showed that Zeb2 overexpression rescued the circ-FOXO3 overexpression-mediated inhibition of TNBC cell migration and invasion (Figure 4I-K). Collectively, our results demonstrate that circ-FOXO3 inhibits TNBC cell proliferation and metastasis by suppressing Zeb2.

Circ-FOXO3 interacts with WHSC1 and inhibits Zeb2 expression

To investigate how circ-FOXO3 regulates Zeb2 expression, the Zeb2 promoter was cloned into a pGL3-basic vector, and its activity was evaluated. Luciferase reporter assay results showed that Zeb2 promoter activity increased dramatically in circ-FOXO3-overexpressing TNBC cells (Supplementary Figure S4), suggesting that circ-FOXO3 might regulate Zeb2 transcription. We then performed a biotin-labeled RNA pull-down, followed by proteomic analysis of the RNA-associated protein complex to identify the protein partner of circ-FOXO3 in TNBC cells (Figure 5A). Mass spectrometry revealed that 69 proteins were
consistently pulled down by biotin-labeled circ-FOXO3 in TNBC cells (Figure 5B, Supplementary Table S4). We identified Wolf-Hirschhorn syndrome candidate 1 (WHSC1) as a potential protein interacting with circ-FOXO3 (Figure 5C). Consistently, the RIP assay indicated the endogenous enrichment of circ-FOXO3 in RNA co-precipitated with the WHSC1 antibody in MDA-MB-231 cells, which was increased by stable overexpression of circ-FOXO3 (Figure 5D). In addition, we performed Dual RNA-FISH and immunofluorescence analyses and found that circ-FOXO3 co-localized with WHSC1 in the cytoplasm of circ-FOXO3-overexpressing MDA-MB-231 and TNBC1937 cells (Figure 5E). Moreover, we fractionated the nuclear and cytosolic extracts from circ-FOXO3-overexpressing MDA-MB-231 and TNBC1937 cells and examined the status of WHSC1 using western blotting. Our results showed decreased nuclear localization of WHSC1 in circ-FOXO3-overexpressing TNBC cells compared to control cells (Figure 5F). WHSC1 encodes a SET domain-containing histone methyltransferase that targets H3K36 [12, 13]. Consistently, circ-FOXO3 overexpression significantly reduced H3K36me2 levels (Figure 5G).

Given that WHSC1-mediated H3K36me2 expression is associated with active gene transcription [14, 15], we investigated whether WHSC1 participates in the regulation of Zeb2. WHSC1 overexpression significantly increased the mRNA and protein expression of Zeb2 (Figure 6A), whereas WHSC1 knockdown decreased Zeb2 mRNA and protein levels (Figure 6B-C). Chromatin immunoprecipitation (ChIP) showed that WHSC1 was significantly enriched at 0.7 to 1.2 (P2 and P3) kilobases upstream of the transcription start site (TSS; Figure 6E). The levels of H3K36me2 modification at the P3 loci in WHSC1-knockdown TNBC cells were significantly lower than those in control cells (Figure 6F). Luciferase reporter analysis showed that ZEB2 promoter activity in TNBC cells was suppressed by WHSC1 shRNA (Figure 6G), indicating that WHSC1 loss resulted in the transcriptional repression of Zeb2 expression. Moreover, we observed a positive correlation between WHSC1 and Zeb2 in breast cancer tissues using Gene Expression Profiling Interactive Analysis (GEPIA; Figure 6H). More importantly, WHSC1 overexpression reversed the promoter activity and expression of Zeb2 in TNBC cells stably transfected with circ-FOXO3 (Figure 6I-K). Collectively, these results indicate that circ-FOXO3 interacts with WHSC1, thereby inhibiting Zeb2 promoter activity and expression.

**Circ-FOXO3 interacts with WHSC1 to inhibit TNBC cell proliferation and metastasis**

To further investigate the interplay between circ-FOXO3 and WHSC1 in the regulation of TNBC progression, we restored WHSC1 expression in circ-FOXO3-overexpressing TNBC cells. We found that WHSC1 did not affect circ-FOXO3 levels in TNBC cells (Figure 7A). Particularly, CCK-8 and colony formation assays revealed that WHSC1 overexpression in circ-FOXO3-overexpressing TNBC cells rescued their proliferation (Figure 7B-D, Supplementary Figure S5). Moreover, WHSC1 overexpression abolished the increase in E-cadherin levels and decreased vimentin levels in TNBC cells stably transfected with circ-FOXO3 (Figure 7E). Furthermore, wound healing and transwell assays showed that restoration of WHSC1 rescued the circ-FOXO3 overexpression-mediated inhibition of TNBC cell migration and invasion (Figure 7F-H). Collectively, our results demonstrate that circ-FOXO3 inhibits TNBC cell proliferation and metastasis by suppressing WHSC1-induced EMT.
Blood circ-FOXO3 serves as a novel biomarker for TNBC metastasis

We further investigated the value of circ-FOXO3 as a novel plasma biomarker of TNBC. We found that circ-FOXO3 expression in the blood of patients with TNBC was significantly lower than that in healthy women (p=0.0005; Figure 8A), indicating that decreased blood circ-FOXO3 levels might correlate with the malignant behavior of TNBC. We further investigated whether circ-FOXO3 expression correlated with metastasis of TNBC; the results showed that low expression of blood circ-FOXO3 was significantly associated with positive lymph node metastasis (p=0.024; Figure 8B). These findings suggest that decreased blood circ-FOXO3 is associated with the aggressive behavior of TNBC and may serve as a biomarker for TNBC metastasis.

Discussion

Breast cancer is a heterogeneous disease with distinct histopathological subtypes and clinical behaviors. TNBC, the most aggressive breast cancer subtype, has drawn considerable attention for its treatment and prognosis owing to the lack of specific biomarkers and therapeutic targets. As a novel class of non-coding RNAs, circRNAs have been implicated in the pathophysiology of cancer and other diseases. Because of their tissue specificity and stable structure, circRNAs show great potential as tumor biomarkers. In this study, we found that circ-FOXO3 may serve as a valuable prognostic biomarker for TNBC.

Increasing evidence indicates that circRNAs are aberrantly expressed in multiple types of tumors and exert tumor-suppressive functions [16-18]. To identify differentially expressed circRNAs in TNBC, we analyzed the profiles of circRNAs in paired TNBC and adjacent normal tissues. The results showed that circ-FOXO3 expression was significantly downregulated in TNBC tissues compared to that in normal and non-TNBC tissues.

Circ-FOXO3 is formed by the exon of FOXO3 and is characterized as a tumor-suppressive gene that encodes a transcription factor with a forkhead DNA-binding domain. Accumulating evidence has demonstrated that circ-FOXO3 is dysregulated in a wide variety of tumors. Circ-FOXO3 is downregulated in non-small cell lung and breast cancers, where it suppresses cell proliferation and invasion [19, 20], whereas it is upregulated in gastric cancer and glioblastoma, where it promotes tumorigenesis [21, 22]. Nonetheless, its expression and role in prostate cancer remain controversial and warrant further investigation [23, 24]. It is unclear why the aberrant expression of circ-FOXO3 exerts distinct roles in different tumors. This may be ascribed to the derivation of different exons in FOXO3. In our study, we verified that circ-FOXO3 was generated from the third exon of FOXO3 and negatively correlated with TNBC prognosis. Further, circ-FOXO3 overexpression suppressed the proliferation and metastasis of TNBC cells. In particular, circ-FOXO3 reversed the EMT phenotype and reduced the expression of the EMT-related transcription factor Zeb2.

The underlying mechanisms by which circRNAs exert their functions and affect tumor development and progression are heterogeneous. Mounting evidence has confirmed that circRNAs are involved in the post-
transcriptional regulation of gene expression by sponging miRNAs, thus preventing miRNAs from binding, and repressing their target mRNA [25, 26]. Currently, most research about circ-FOXO3 is focused on its miRNA sponging properties [19, 21, 24]. Studies have shown that circRNAs function as protein sponges or inhibitors. They may scaffold different proteins in close proximity or recruit them to distinct subcellular compartments [27, 28]. circ-FOXO3 facilitated E3 ubiquitin ligase MDM2-mediated mutant p53 ubiquitination and degradation by binding p53 and MDM2 together, thereby triggering apoptosis in breast cancer cells [20]. To explore the potential protein partner of circ-FOXO3, we performed RNA pull-down and proteomic analyses of TNBC cells. We verified that circ-FOXO3 interacts with WHSC1, inducing its cytoplasmic localization.

Histone methylation frequently occurs at lysine (K) or arginine (R) residues and exerts positive or negative effects on gene transcription. WHSC1 is a histone methyltransferase that specifically catalyzes di-methylation at lysine 36 of histone H3 (H3K36me2) [29]. This modified form of H3K36 is usually concentrated in regulatory regions closely related to active transcription [30-32]. Previous studies have reported that WHSC1 is highly expressed in multiple tumor types and is associated with poor clinical outcomes [33-35]. Recently, convincing evidence has confirmed that WHSC1 promotes tumor progression and metastasis by regulating genes that contribute to EMT [36, 37]. Abundant WHSC1 expression was detected in TNBC cells. We speculate that WHSC1 may exert its function by modulating the expression of EMT-related factors. Indeed, WHSC1 enhances Zeb2 expression by inducing H3K36me2 methylation of its promoter. Moreover, circ-FOXO3 inhibited TNBC progression by inhibiting WHSC1 expression. Nevertheless, the mechanisms of circ-FOXO3 downregulation and WHSC1 upregulation in TNBC cells require further investigation.

CircRNAs are typically expressed in a tissue- or cell-specific manner [38, 39]. In particular, they have longer half-lives and are not readily degraded by RNases. Therefore, circRNAs are potential biomarkers for cancer diagnosis and prognosis. Besides, earlier studies have identified candidate circRNAs in biofluids such as plasma, urine, and saliva of patients with cancer [40-42]. Therefore, we detected the expression of circ-FOXO3 in the plasma of patients with TNBC. The results showed that the levels of circ-FOXO3 were significantly lower in patients with TNBC than that in healthy women. Particularly, blood circ-FOXO3 levels were negatively correlated with TNBC patients' metastasis, suggesting blood circ-FOXO3 as a biomarker for TNBC metastasis.

**Conclusion**

In summary, our study revealed that circ-FOXO3 expression was significantly lower in TNBC tissues and blood samples. circ-FOXO3 overexpression suppressed the proliferation, invasion, and metastasis of TNBC cells both *in vitro* and *in vivo*. Circ-FOXO3 inhibited TNBC progression by interacting with WHSC1 and inhibiting the WHSC1-H3K36me2-Zeb2 signaling pathway (Figure 8C). Conclusively, circ-FOXO3 may serve as a novel predictive biomarker and therapeutic target in TNBC.

**List of abbreviations**
ChIP, Chromatin immunoprecipitation;
CircRNA, Circular RNAs;
EMT, Epithelial-mesenchymal transition;
ER, Estrogen receptor;
FISH, Fluorescence in situ hybridization;
GEPIA, Gene Expression Profiling Interactive Analysis;
HER2, Human epidermal growth factor receptor 2;
PR, Progesterone receptor;
RT-qPCR, real-time quantitative RT-PCR;
TNBC, Triple-negative breast cancer;
TSS, Transcription start site;
WHSC1, Wolf-Hirschhorn syndrome candidate 1

Declarations

Ethics approval and consent to participate

All experimental protocols were approved by the Ethics Committee of Affiliated Cancer Hospital & Institute of Guangzhou Medical University and performed in accordance with the relevant guidelines and regulations. Written informed consents were obtained from all patients. All animal experiments were approved by the Institutional Animal Care and Use Committee of Guangzhou Medical University (Guangzhou, China).

Availability of data and materials

The datasets used and/or analyzed during the current study are available within the manuscript and its supplementary information files.

Competing interests

The authors declare no potential conflicts of interest.

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Authors' contributions

H.L., Y.Y., Z.L., C.D., and K.L. designed the experiments, analyzed the data, and drafted the manuscript and figures. Z.S., C.D., Q.H., Y.M., Lin.X., Lv.X., L.P., W.S., and K.S. detected the cells' biological function, performed IHC analyses and animal experiments. All authors read and approved the final manuscript.

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**Figures**
Figure 1

Circ-FOXO3 is downregulated in TNBC tissues and cells. (A) Clustered heatmap of differentially expressed circRNAs in three pairs of human TNBC tissues and adjacent normal tissues. (B) The genomic loci of circ-FOXO3, RT-PCR indicating the detection of circ-FOXO3, with further validation by Sanger sequencing. (C) RT-qPCR analysis for the abundance of circ-FOXO3 and its linear counterpart after treatment with RNase R in MDA-MB-231 cells. (D) Cellular localization of circ-FOXO3 in MDA-MB-231 cells was detected by subcellular fractionation assay. (E) The expression of circ-FOXO3 in adjacent normal tissues and tumor tissues of TNBC was detected by RT-qPCR. (F) The expression of circ-FOXO3 in TNBC tumor tissues and non-TNBC tumor tissues was detected by RT-qPCR. (G) The expression of circ-FOXO3 in breast cancer cell lines and normal breast epithelial cells was detected by RT-qPCR. (H) Representative images showed the expression of circ-FOXO3 in TNBC tissues measured by FISH. (I) The association between circ-FOXO3 expression and prognosis of TNBC patients was analyzed by Kaplan–Meier survival analysis. *p < 0.05, **p < 0.01.

Figure 2
Circ-FOXO3 inhibits TNBC cells proliferation. (A) Circ-FOXO3 overexpression plasmid pLCDH-circ-FOXO3 was constructed. (B) MDA-MB-231, BT549 and HCC1937 cells were transfected with pLCDH-circ-FOXO3 or control vector, the expression of circ-FOXO3 was detected by RT-qPCR. (C-D) MDA-MB-231, BT549 and HCC1937 cells were transfected with pLCDH-circ-FOXO3 or control vector, cell proliferation was measured by colony formation assay. (E) MDA-MB-231 and BT549 cells were transfected with pLCDH-circ-FOXO3 or control vector, cell proliferation was measured by CCK-8 assay. (F) Cell cycle analysis of MDA-MB-231 and BT549 cells transfected with pLCDH-circ-FOXO3 or control vector by flow cytometer. (G-I) MDA-MB-231 cells transfected with pLCDH-circ-FOXO3 or control vector were subcutaneously inoculated into nude mice for generating xenograft tumors; at the end of experiment, tumors were excised (G) and weighted (I); the tumor size was measured at indicated time (H); tumor tissues were fixed, sectioned, and placed on slides. Tumor specimens were subjected to IHC staining for Ki-67 (J). *p < 0.05, **p < 0.01.

Figure 3
Circ-FOXO3 inhibits TNBC cells metastasis. (A-D) MDA-MB-231, BT549 and HCC1937 cells were transfected with pLCDH-circ-FOXO3 or control vector, cell migration ability was measured by wound-healing assay (A), cell invasion ability was measured by transwell assay (B-C), the expression levels of EMT-related factors Vimentin and E-cadherin were detected by western blotting (D). (E-G) MDA-MB-231 cells transfected with pLCDH-circ-FOXO3 or control vector were inoculated into nude mice by tail vein to detect lung metastasis, at the end of experiment, the lungs of nude mice were dissected (E), Representative images of lung sections by HE staining (F), quantification of metastatic lung nodules (G). **p < 0.01.
Figure 4

Circ-FOXO3 suppressed TNBC cell proliferation and metastasis via inhibition of Zeb2. (A-B) Heat map (A) and Volcano plots (B) showing the differentially expressed genes in MDA-MB-231 cells stably transfected with control vector (NC) or pLCDH-circ-FOXO3. (C) The expression of Zeb2 in MDA-MB-231 cells stably transfected with empty vector (NC) or pLCDH-circ-FOXO3. (D-E) MDA-MB-231, BT549 and HCC1937 cells were transfected with pLCDH-circ-FOXO3 or control vector, the expression levels of Zeb2 were analyzed by RT-qPCR (D) and western blotting (E). (F) MDA-MB-231 or BT549 cells were co-transfected with pLCDH-circ-FOXO3 or control vector and pCMV or pCMV-Zeb2, the protein levels of Zeb2 were measured by western blotting. (G-H) MDA-MB-231 or BT549 cells were co-transfected with pLCDH-circ-FOXO3 or control vector and pCMV or pCMV-Zeb2, cell proliferation was measured by CCK8 assay (G) and colony formation assay (H). (I-K) MDA-MB-231 or BT549 cells were co-transfected with pLCDH-circ-FOXO3 or control vector and pCMV or pCMV-Zeb2, cell migration ability was measured by wound-healing assay (I), cell invasion ability was measured by transwell assay (J-K). *p < 0.05, **p < 0.01.

Figure 5

Circ-FOXO3 interacts with WHSC1 and inhibits WHSC1 activity. (A) Schematic illustration showing the procedure for identifying the circ-FOXO3-interacting protein by RNA pull-down and mass spectrometry analysis. (B) Proteins pulled down by biotin-labeled circ-FOXO3 in TNBC cells. (C) Mass spectrometry of WHSC1 peptide. (D) RIP assay of circ-FOXO3 coprecipitated by WHSC1 in MDA-MB-231 cells. (E) Dual
RNA-FISH and immunofluorescence analysis showing the co-localization of circ-FOXO3 and WHSC1 in MDA-MB-231 and HCC1937 cells. (F-G) MDA-MB-231 and HCC1937 cells were transfected with pLCDH-circ-FOXO3 or control vector; subcellular expression of WHSC1 was detected by subcellular fractionation assay (F), the expression of WHSC1 and H3K36me2 were detected by western blotting (G). **p < 0.01.

Figure 6

WHSC1 mediates H3K36me2 modification promotes ZEB2 transcription. (A-B) MDA-MB-231 and BT549 cells were transfected with WHSC1-overexpressing vector or control vector, the expression of WHSC1 and Zeb2 were detected by western blotting (A) and RT-qPCR (B). (C-D) MDA-MB-231 and BT549 cells were transfected with WHSC1 shRNA or control shRNA, the expression of WHSC1 and Zeb2 were detected by western blotting (C) and RT-qPCR (D). (E) Upper, schematic diagram of the primer pair location in Zeb2 promoter. Lower, CHIP analyses of Zeb2 promoter in MDA-MB-231 cells by use of antibody of WHSC1 and H3K36me2. (F) CHIP analyses of Zeb2 promoter in MDA-MB-231 cells transfected with WHSC1 shRNA or control shRNA by use of antibody of H3K36me2. (G) MDA-MB-231 and BT549 cells were
transfected with WHSC1 shRNA or control shRNA followed by transfected with a luciferase reporter construct containing the Zeb2 promoter, the relative luciferase activities were analyzed. (H) The correlation between WHSC1 and Zeb2 in breast cancer was assayed via Gene Expression Profiling Interactive Analysis. (I-K) MDA-MB-231 and BT549 cells were co-transfected with pLCDH-circ-FOXO3 and WHSC1-overexpressing vector, the Zeb2 promoter luciferase activities were analyzed (I), the expression of Zeb2 was measured by RT-qPCR (J) and western blotting (K). *p < 0.05, **p < 0.01.

Figure 7
Circ-FOXO3 interacts with WHSC1 to inhibit TNBC progression. (A) MDA-MB-231 cells were transfected with WHSC1-overexpressing vector or control vector, the expression of circ-FOXO3 were detected by RT-qPCR. (B-D) MDA-MB-231 cells were co-transfected with pLCDH-circ-FOXO3 and WHSC1-overexpressing vector, cell proliferation was measured by CCK8 assay (B) and Colony formation assay (C-D). (E-H) MDA-MB-231 or BT549 cells were co-transfected with pLCDH-circ-FOXO3 and WHSC1-overexpressing vector, the expression of Vimentin, E-cadherin, WHSC1 and H3K36me2 were detected by western blotting (E), cell migration ability was measured by wound-healing assay (F), cell invasion ability was measured by transwell assay (G-H). *p < 0.05, **p < 0.01.
Figure 8

Blood circ-FOXO3 serves as a biomarker in TNBC. (A) RT-qPCR analyses of circ-FOXO3 expression level in blood of TNBC patients (n=38) and normal healthy women (n=10). (B) RT-qPCR analyses of circ-FOXO3 expression level in blood of TNBC patients with LN metastasis (n=27) or non-LN metastasis (n=11). (C) The mechanisms underlying circ-FOXO3–promoted TNBC progression.
Supplementary Files

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