1. Introduction

Breast cancer (BC) belongs to a recurrent malignancy, as well as the major cause of cancer-linked mortality in females all over the world [1, 2]. Despite varying degrees of improvement in diagnosis, prognosis, and treatment, the recurrence together with mortality rates in women with BC remain high [3, 4]. Thus, efficient diagnostic or therapeutic approaches are required to understand and identify vital molecular targets engaged in the progression of BC.

Circular RNAs (circRNAs) belong to a novel category of non-coding RNAs that generate a connecting loop at 3’ and 5’ ends [5]. Since circRNA is widely expressed in mammals [6], its potential role in human diseases has shed light on researching their mechanism [7]. Recently, numerous literatures have probed the potential of different novel circRNAs in BC [8]. For example, circRNA_0025202 affects BC progression through the miR-182-5p-FOXO3a axis [9]. CircCDYL regulates the miR-1275-ATG7/ULK1-autophagic axis to facilitate BC progression [10]. CircRNF20 increases BC tumorigenesis as well as the Warburg effect via miR-487a-HIF-1α-HK2 [11].

Herein, the potential of a novel circRNA hsa_circ_0006743 (circ_JMJD1C) in BC progression was probed. Circ_JMJD1C has been registered to be upregulated in BC and contributed to BC progression [12]. Circ_JMJD1C is derived from its host gene Jumonji Domain Containing 1C (JMJD1C), which is located in 10q21.3, and it is suggested to possess an oncogenic role in BC [12]. Accumulating reports have elucidated the vital role of JMJD1C in cancer [13]. Intriguingly, a similar circRNA, circ-TFF1, has been reported to positively regulate its host gene TFF1 in BC by competitively combining with miR-326 [14]. This phenomenon has relied on their closely related location in the human genome. Our findings utilized a similar hypothesis and investigated the role of circ_JMJD1C and its relation to JMJD1C in BC progression. We proved that circ_JMJD1C/miR-182-5p/JMJD1C axis had a tumor-promoting potential in BC, providing a promising biomarker for the therapy of BC patients in the near future.

2. Materials and methods

2.1. Sample collection

BC tissues (n = 14), para-carcinoma tissues (n = 12), as well as adjacent normal tissues (n = 11), were provided by 14 patients with this cancer in Zigong First People's Hospital. After biopsies or sample resection, all samples...
were maintained in tubes supplemented with the RNase inhibitor and stored in liquid nitrogen. All samples were collected after signing an informed consent form. None of the enrolled patients had received any therapy before this study. The present experiments were approved by the ethical committee of Zigong First People's Hospital.

2.2. Cell culture

Procell Life Science & Technology Co., Ltd provided BC cell lines (MCF-7, BT-549, MDA-MB-453, and MDA-MB-231), as well as the normal breast cells (MCF-10A). DMEM (Gibco, USA) supplementing with 10% FBS (Gibco) was implemented for cell culture at 37°C with 5% CO₂.

2.3. Gene transfection

GenePharma (Shanghai, China) provided sh-circ-JMJD1C#1/2/3, miR-182-5p inhibitor, as well as sh-JMJD1C#1 together with the respective controls. Lipofectamine 3000 (Thermo Fisher Scientific, USA) was implemented for cell transfection.

2.4. qRT-PCR

Firstly, total RNA extraction was implemented using TRizol reagent (Thermo Fisher Scientific), followed by reverse transcription using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Next, qPCR was determined using SYBR Green PCR Master Mix (Applied Biosystems) and following the \( 2^{-\Delta\Delta CT} \) method. Gene expression was normalized to GAPDH or U6.

2.5. Western blot

Total protein from cell or tissue samples was extracted using RIPA buffer (#ab156034, Abcam, Cambridge, UK). A total amount of extracted proteins (20 μg) was electrophoresed in 10% SDS-PAGE and then shifted onto a PVDF membrane (#E-BC-R266, Elabscience). Membranes were sealed in 5% fat-free milk for one hour and followed by incubation with each designated primary antibody, containing anti-Bcl-2 (#ab32124, Abcam), anti-Bax (#ab32503, Abcam), anti-cleaved caspase-3 (#ab32042, Abcam), anti-caspase-3 (#ab32351, Abcam), anti- cleaved caspase-3 (#ab32042, Abcam), anti- E-cadherin (#ab40772, Abcam), anti-N-cadherin (#ab76011, Abcam), anti-Fibronectin (#ab2413, Abcam), anti-Vimentin (#ab92547, Abcam), anti-JMJD1C (#ab130922, Abcam), anti-GAPDH (#ab8245, Abcam) overnight at 4°C. Next, membranes were washed thrice and then treated with the corresponding Rabbit Anti-Mouse IgG H&L (HRP) (#ab6721, Abcam). The iBright Analysis Software (Thermo Fisher Scientific) was employed to determine the protein band intensities with GAPDH as an internal control.

2.6 TUNEL assay

The cellular apoptosis rate was determined using the TUNEL assay kit (#E-CK-A322, Elabscience). All treated cells underwent fixation using 4% paraformaldehyde at 4°C. Next, the cells were stained using the TUNEL kit, while the nuclei of cells were stained using DAPI (#ab285390, Abcam), followed by counting using a BX53 fluorescence microscope.

2.7. CCK-8 assay

The cell viability was measured using a Cell Counting Kit-8 (#ab228554, Abcam) as instructed by the manufacturer. Approximately \( 2 \times 10^3 \) cells/well were planted into 96-well plates. Each well was treated with CCK-8 solution (10 μL) for 2 hours of incubation. Lastly, The OD values were measured using a microplate reader at 450 nm.

2.8. EdU assay

The proliferation of BC cells was assessed using the EdU assay detection kit (#ab222421, Abcam). 5000 cells/well were plated in 96-well plates. Following forty-eight hours of incubation, the plates were treated with 50 μmol/L EdU labeling media and then incubated for an additional two hours. Next, the cells were treated with 0.5% Triton X-100 and 4% paraformaldehyde then treated with an anti-EdU working solution. DAPI was used to stain the nucleus. The proportion of EdU-positive cells was quantified using a fluorescent microscope. The images were taken from five random fields.

2.9. Transwell assay

Cell invasion assay was conducted using 24-well chambers with 8 μm sized-pores (Sigma-Aldrich). BC cells (5 \( \times 10^4 \) cells/well) were cultured in serum-free media and placed in the upper chamber pre-coated with matrigel (Corning). The lower chamber was loaded with a culture medium supplemented with 10% FBS. After 24 h incubation, the upper chambers were removed, and the invasive cells were fixed with methanol and then subjected to staining. At last, the cells were examined and counted under a BX53 microscope at 200× magnification power.

2.10. Wound healing assay

BC cells (5 \( \times 10^3 \)) were placed in 6-well plates. The wound induction was made via a sterile 200 μL plastic pipette tip to scrap the cell layer. The cells were further supplemented with a culture medium containing 1% FBS and incubated for 36 h. Next, all plates were examined under a BX53 microscope at 50× magnification power.

2.11. Cellular fractionation

The cytoplasm and nucleus of BC cells were separated using a PARIS kit (Thermo Fisher Scientific) in order to segregate the cytoplasmic and nuclear RNA. The concentration of fractionated RNA was assessed using qRT-PCR.

2.12. Fluorescent in-situ hybridization (FISH)

A FISH kit (#F32949, Thermo Fisher Scientific) was utilized for this experiment, and the FISH probes were designed from RIBOBIO (Guangzhou, China). BC cells were fixed in 4% formaldehyde and subsequently dehydrated with ethanol. Next, cells were treated with hybridization buffer mixed with the FISH probes for incubation. After washing and staining using DAPI solution, the cells were examined under a fluorescence microscope (BX63, Olympus, Tokyo, Japan).

2.13. RNA immunoprecipitation (RIP) assay

The AGO2-RIP assay was implemented using EZ-Magna RIP Kit (Sigma-Aldrich). Briefly, the lysates of BC cells were incubated in RIP buffer containing magnetic beads-conjugated human anti-Ago2 or nonspecific mouse IgG antibodies (both from Abcam). Precipitation was conducted using magnets, then the immunoprecipitants were digested by proteinase K, and the released RNAs
were analyzed by qRT-PCR.

2.14. RNA pull-down assay

Briefly, BC cell lysates were incubated with biotin-(Bio)-labeled circ-JMJD1C probes (RIBOBIO) at 25°C for two hours. Streptavidin-coupled Dynabeads (#11205D, Thermo Fisher Scientific) were utilized to capture the circ-JMJD1C-linked miRNA complexes. The beads/RNA complexes were incubated for one hour at 25°C in RIPA wash buffer (#R0278, Sigma-Aldrich) containing proteinase K. Successful RNA pull-down was determined using qRT-PCR analysis.

2.15. Luciferase reporter assay

The wild-type or mutated circ-JMJD1C contains miR-182-5p binding sites obtained from Circular RNA Interactome. The constructs were subcloned into the pmirGLO Vector (Promega). Then, miR-182-5p mimic or its control, circ-JMJD1C-WT, circ-JMJD1C-MUT, circ-JMJD1C overexpression plasmids, JMJD1C overexpression plasmids, or the corresponding vectors were co-transfected into BC cells. Twenty-four hours post-transfection, the luciferase activity was evaluated using the Dual-Glo® Luciferase Assay System (Promega).

2.16. ChIP assay

ChIP assay was conducted using a ChIP kit (cat. no. 26157, Thermo Fisher Scientific Inc., USA) according to the manufacturer’s protocols. Briefly, cells were fixed in 1% formaldehyde and resuspended in lysis buffer. Then shearing chromatin was performed by adding adding enzymatic shearing cocktail and stopped by supplementing with EDTA. After pre-cleared with Protein A/G agarose beads, the supernatants were incubated with anti-JMJD1C (3100, 1: 50, CST, USA) or anti-IgG (ab172730, 1: 30, Abcam, USA). The collected chromatin was eluted and proteinase K. ChIP-enriched DNA was determined using PCR.

2.17. Bioinformatics analysis

The binding sites between miR-182-5p and circ-JMJD1C/JMJD1C were analyzed by using the online database Starbase3.0 (http://starbase.sysu.edu.cn/). The expression of epithelial-to-mesenchymal transition transcription factors (EMT-TFs), correlation between SOX4 and JMJD1C expression as well as survival rates of breast cancers were analyzed by using the online database GEPIA (http://gepia.cancer-pku.cn/).

2.18. Tumor formation and in vivo metastasis assay

Three male BALB/C nude mice (4-6 weeks old) were commercially bought from Nanjing Aibei Biotechnology Co., Ltd. BT-549 cells (1×10^6) harbors sh-NC or sh-circ-JMJD1C plasmids were injected into the posterior side of the mice. Each mice group contained five mice. The xenograft tumor growth was determined every two days. Four weeks later, the mice were sacrificed, and the lungs were harvested. The metastatic nodules were observed via IHC analysis. The present study was conducted and strictly followed up with the requirements of the National Institutes of Health (NIH) for animal experiments and obtained approval from the animal care committee of Zigong First People's Hospital.

2.19. Statistical analysis

The present study utilized SPSS together with GraphPad Prism 5 software for statistical analysis. Student’s t-test or chi-square test was employed to estimate differences between 2 groups, whereas one-way analysis of variance (ANOVA) was used to estimate differences between 3 or more groups. The Pearson correlation coefficient was utilized to determine the relation between genes. P < 0.05 was significant. All presented data were expressed as means ± standard error of the mean (SEM).

3. Results

3.1. Circ-JMJD1C and JMJD1C were highly expressed in BC

It has been registered that distinct circRNAs were aberrantly expressed in BC (12). Among them, circJMJD1C possessed the most significant fold changes (12). The UCSC database indicated that circ-JMJD1C (chr10 63,465,896) was formed by the exons of its host gene JMJD1C (Exon 1, chr10: 63,316,601), which implied a potential management of JMJD1C by circ-JMJD1C (data not shown). Additionally, the cyclic structure of circ-JMJD1C was certified as it was only amplified by divergent primers (Figure 1A). Subsequently, the expression of circ-JMJD1C and linear JMJD1C in healthy tissues, para-carcinoma tissues, as well

Fig. 1. Circ-JMJD1C and JMJD1C are upregulated in BC. (A) Divergent primer PCR verified the circular structure of circ-JMJD1C. (B-C) qRT-PCR outcomes of circ-JMJD1C and JMJD1C expression in healthy, para-carcinoma, as well as tumor tissues. (D-E) qRT-PCR examined circ-JMJD1C and JMJD1C expression in tumor tissues in T1, T2, as well as T3 stages. (F) Pearson’s correlation analysis of circ-JMJD1C and JMJD1C. (G) Circ-JMJD1C expression in BC cells along with MCF-10A from qRT-PCR. (H) qRT-PCR tested the silencing efficiency of circ-JMJD1C in BC cells. (I-J) JMJD1C expression in BC cells after circ-JMJD1C silence from qRT-PCR together with Western blot. (K-L) FISH and subcellular fractionation assays tested the localization of circ-JMJD1C in BC cells. *P < 0.05, **P < 0.01, ***P < 0.001.
as BC samples were shown as low, middle and high (Figure 1B-C). Notably, the levels of circ-JMJD1C and linear JMJD1C were enhanced accompanied by BC progression (Figure 1D-E). Significantly, in BC tissues, a positive relation between JMJD1C and circ-JMJD1C expression was observed (Figure 1F).

In comparison with normal cells, circ-JMJD1C was highly expressed in BC cells (Figure 1G). Then, circ-JMJD1C was knocked down in two BC cells. As shown in Figure 1H, all shRNAs constructs targeting circ-JMJD1C remarkably reduced circ-JMJD1C expression in both cells. Notably, sh-circ-JMJD1C#1 was chosen for subsequent analysis because of its highest silencing efficiency. Expectedly, circ-JMJD1C inhibition resulted in decreased JMJD1C expression at mRNA along with protein levels (Figure 1I, J). Additionally, the FISH assay indicated the distribution of circ-JMJD1C was mainly in the cytoplasm (Figure 1K), which was also confirmed by qPCR from the fractionated mRNA profile in the cytoplasm and nucleus (Figure 1L).

### 3.2. Circ-JMJD1C knockdown hindered cell proliferation and promoted breast cancer cell apoptosis

The loss-of-function experiments were arranged to uncover circ-JMJD1C potential in BC. CCK-8 assay demonstrated circ-JMJD1C depletion repressed the viability of two selected BC cells (Figure 2A). Western blot results displayed circ-JMJD1C reduction suppressed Bcl-2 expression whereas elevated of Bax and cleaved caspase-3 levels (Figure 2B). Consistently, the EdU assay manifested BC cell proliferation was reduced upon circ-JMJD1C down-regulation (Figure 2C). TUNEL assay demonstrated that BC cell apoptosis was elevated in two selected BC cells after transfection with sh-circ-JMJD1C #1 (Figure 2D). The cell motility declined after circ-JMJD1C knockdown (Figure 2E). Similarly, the transwell assay showed circ-JMJD1C knockdown repressed cell invasion (Figure 2F). Western blot results confirmed the enhanced expression of E-cadherin along with suppression of N-cadherin, MMP2, and MMP9 levels when circ-JMJD1C was knocked down (Figure 2G). Immunofluorescence assay expounded significant decrease of N-cadherin and MMP-2 expression upon circ-JMJD1C downregulation in BC cells (Figure 2H).

### 3.3. Circ-JMJD1C upregulated JMJD1C expression through sponging miR-182-5p

We discovered that miR-182-5p potentially targets both circ-JMJD1C and JMJD1C (Figure 3A). Then, miR-182-5p was discovered to be low-expressed in all BC cells (Figure 3B). Similarly, we verified that miR-182-5p expression was also drastically reduced in tumor tissues relative to healthy and para-carcinoma specimens (Figure 3C). Besides, miR-182-5p expression was negatively related to circ-JMJD1C expression (Figure 3D). The binding sites of miR-182-5p and circ-JMJD1C or JMJD1C were revealed in Figure 3E. The outcomes of the luciferase reporter assay manifested that overexpressed miR-182-5p could decline the luciferase activity of JMJD1C-WT together with circ-JMJD1C-WT, but did not impact that of JMJD1C-MUT and circ-JMJD1C-MUT (Figure 3F). Besides, it was unraveled that circ-JMJD1C, miR-182-5p, and JMJD1C were abundant in the anti-Ago2 antibody precipitated compounds from RIP assays (Figure 3G). Meanwhile, RNA pull-down experiments suggested that circ-JMJD1C and JMJD1C were pulled down by miR-182-5p (Figure 3H). MiR-182-5p expression was also negatively related to JMJD1C expression (Figure 3I). Additionally, silenced circ-JMJD1C inhibited JMJD1C expression while promoting the miR-182-5p expression in two BC cells (Figure 3J). Of note, we confirmed that JMJD1C protein expression was inhibited by circ-JMJD1C knockdown and then recovered after cosuppression of miR-182-5p (Figure 3K).

### 3.4. Circ-JMJD1C/JMJD1C complex epigenetically activates EMT-transcription factor (TF) SOX4

EMT-TF is the core regulator of EMT machinery. The alteration of EMT-TF may promote or reverse the processes of EMT. Therefore, we determined the expression of EMT-TF in breast cancer. As shown in Figure 4A, we found that SOX4 expression was significantly increased in breast cancer patients, while TWIST1 and ZEB1 expression were significantly downregulated. Moreover, we determined the expression of SOX4, TWIST1 and ZEB1. We found that the mRNA expression of SOX4, TWIST1 and ZEB1 was significantly upregulated by overexpressed circ-JMJD1C (Figure 4B), while decreased by circ-JMJD1C knockdown. Moreover, SOX4 expression was positively correlated with that of JMJD1C (Figure 4C). High expression of SOX4 predicted poor survival rates in...
The roles of circ_JMJD1C in breast cancer.

The long-term run (Figure 4D). Figure 4E shows the binding sites between JMJD1C and SOX4 (Up) and the binding motif of SOX4 (Down). We found that cotransfection with circ JMJD1C/JMJD1C markedly enhanced the transcription activation of SOX4 (Figure 4F). Moreover, mutation in the binding sites markedly abrogated the regulation of circ-JMJD1C and miR-182-5p expression in BC cells after circ-JMJD1C silence. (K) Western blot examined JMJD1C expression in BC cells transfected with sh-circ-JMJD1C, sh-circ-JMJD1C+miR-182-5p inhibitor or sh-circ-JMJD1C+pcDNA-circ-JMJD1C. *P < 0.05, **P < 0.01, ***P < 0.001.

3.5. Knockdown of circ-JMJD1C impeded BC tumor growth in vivo

Animals were injected with circ-JMJD1C-silenced BC cells. We found the tumors were shrunken and distinctly more diminutive due to circ-JMJD1C decrease (Figure 5A-C). Simultaneously, we discovered that circ-JMJD1C together with JMJD1C expressions were declined upon miR-182-5p overexpression in tumors from mice injected with circ-JMJD1C-silenced BC cells compared to mice injected with control cells (Figure 5D). Additionally, the western blot analysis displayed that the silenced circ-JMJD1C suppressed JMJD1C, Ki67, Bcl-2, MMP2, and MMP9, as well as N-cadherin levels, whereas elevated Bax together with E-cadherin levels (Figure 5E). Consistently, IHC analysis showed circ-JMJD1C silence declined Ki67 along with N-cadherin levels, whereas elevated E-cadherin level (Figure 5F-G).

4. Discussion

In this study, circRNA_104348 was overexpressed in breast cancer patients and cells. Moreover, high levels of circRNA_104348 were associated with tumorigenesis and advanced stages. Interestingly, circRNA_104348 knockdown suppressed the proliferation and EMT of breast cancer cells, while promoting tumor cell apoptosis. Moreover, circRNA_104348 sponged miR-182-5p to upregulate its cognate gene JMJD1C, the knockdown of which promotes suppressed the aggressiveness of breast cancer cells. Therefore, circRNA_104348/miR-182-5p/JMJD1C may be a potential target for breast cancer.

Non-coding RNAs comprise broad types of RNAs,
The roles of circ_JMJD1C in breast cancer.

In conclusion, the present study demonstrated that circ_JMJD1C, derived from the host gene JMJD1C, induces BC oncogenesis from miR-182-5p-JMJD1C-SOX4 axis. These findings suggest that miR-182-5p may function as an anti-tumor miRNA. However, the roles of miR-182-5p in tumorigenesis are contradictory. High expression of miR-182-5p predicts advanced tumor-node-metastasis (TNM) stages and lymph node metastasis in patients with non-small cell lung cancer [22]. miR-182-5p promotes tumor cell growth in liver cancer [23]. miR-182-5p accelerates radioresistance in nasopharyngeal carcinoma [24]. These findings dictate that miR-182-5p may also function as an onco-miRNA. Therefore, identifying the exact roles of miR-182-5p in breast cancer is of vital importance. In our study, miR-182-5p was downregulated in breast cancer patients and cells. However, miR-182-5p deficiency promoted the proliferation and EMT of breast cancer cells, suggesting that miR-182-5p may function as an anti-tumor miRNA in breast cancer.

circRNAs function as ceRNA to regulate gene expression via sponging miRNAs. For instance, circSEPT9/miR-637/LIF axis promotes the carcinogenesis and development of triple-negative breast cancer [25]. circRNF20 drives the Warburg effect and tumorigenesis in breast cancer through regulating miR-487a/HIF-1α/HK2 [11]. In this study, circ_JMJD1C-mediated upregulation of JMJD1C via binding to miR-182-5p, thereby affecting the cellular activity of BC. JMJD and its isoforms were explicitly observed to be promoted and engaged in the development of BC [13]. In this study, JMJD1C was overexpressed in breast cancer cells and tissues. circ_JMJD1C expression was positively correlated with that of JMJD1C in breast cancer tissues. Moreover, circ_JMJD1C was majorly localized in the cytoplasm of BC cells, implying that circ_JMJD1C may participate in the ceRNA network by modulating JMJD1C.

Epithelial-mesenchymal transition (EMT) is characterized by the degradation of epithelial functions and the acquisition of mesenchymal features [26], which is accompanied by the downregulation of E-cadherin and upregulation of N-cadherin, Vimentin, Fibronectin, etc. EMT is associated with tumor initiation, growth, immunosurveillance, and metastasis [27-29]. Activated EMT signaling promotes the metastasis and chemoresistance of breast cancer [30, 31]. EMT-inducing transcription factors (EMT-TFs) play a key role in tumorigenesis. EMT-TFs accelerate the processes of EMT via activating mesenchymal genes and inhibiting epithelial genes [32]. The nuclear interaction of EMT-TFs with larger protein complexes in epigenetic reprogramming during the pathogenesis of breast cancer has attracted increasing attention in recent years. For instance, high levels of ZEB1,2 drive epithelial-mesenchymal plasticity (EMP) in estrogen receptor-positive breast cancer dormancy [33]. Nuclear localization of TWIST1 induces breast tumor invasion and metastasis in vivo [34]. In this study, we found that SOX4 was overexpressed in breast cancer. Moreover, high expression of SOX4 was associated with poor survival of breast cancer patients. circ_JMJD1C epigenetically upregulated SOX4, which induced the activation of mesenchymal genes and promoted the EMT of breast cancer cells.

Informed consent
The authors report no conflict of interest.
Availability of data and material
We declared that we embedded all data in the manuscript.

Authors' contributions
XR conducted the experiments and wrote the paper; LH, ZL, YS and MY analyzed and organized the data; CH conceived, designed the study and revised the manuscript.

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References

210
The roles of circ_JMJD1C in breast cancer.


