ZEB1-Mediated Transcriptional Upregulation of circWWC3 Promotes Breast Cancer Progression through Activating Ras Signaling Pathway

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INTRODUCTION
Zinc finger E-box binding homeobox 1 (ZEB1), which is a transcription factor, has been widely recognized as an important driver of tumor growth and metastasis. However, nothing is known about ZEB1-regulated circular (circ)RNAs in cancer. In the current study, we evaluated the function of a novel ZEB1-regulated circRNA derived from the WWC3 gene locus, circWWC3 in breast cancer progression.

Circular RNAs (circRNAs) are a novel class of noncoding RNAs that are derived from precursor (pre)-mRNA back splicing and are covalently closed transcripts and therefore, are highly stable compared to their linear types.1–10 Accumulating evidence demonstrated that circRNAs are involved in the development of various carcinomas through acting as microRNA (miRNA) sponges, forming RNA-protein or RNA-RNA complexes and regulating targeted gene splicing and transcription.11–15 It has also been demonstrated that circRNAs are generated cotranscriptionally and that canonical pre-mRNA splicing can compete with circularization of exons.16 For instance, estrogen receptor β (ERβ) has been reported to transcriptionally suppress circATP2B1 expression, leading to reduced microRNA (miR)-204-3p, which increased fibronectin 1 (FN1) expression and enhanced clear cell renal cell carcinoma (cRCC) cell invasion.17 Transcription factor androgen receptor (AR) was reported to suppress circHIAT1 expression, which promoted cRCC development.18 Twist1 binds the Cul2 promoter to activate its transcription and selectively promote expression of Cul2 circRNA but not mRNA, which regulated vimentin expression and promoted hepatocellular carcinoma progression.19 As a transcription factor, how ZEB1 regulates circRNA expression and functions in breast cancer progression are still poorly understood.

In the present study, we found ZEB1 promotes the transcription of pre-WWC3 through binding to the WWC3 promoter and upregulates circWWC3 expression but not WWC3 mRNA expression. circWWC3 was highly expressed in breast cancer tissues and is associated with the poor prognosis of breast cancer patients. Functionally, the circWWC3 significantly suppresses the proliferation, migration, and invasion of breast cancer cells. Mechanically, circWWC3 upregulates multiple oncogenes' expression of the Ras signaling pathway through acting as the sponge of microRNA (miR)-26b-3p and miR-660-3p. Moreover, short hairpin (sh)RNA-mediated knockdown of circWWC3 partially antagonized ZEB1-mediated breast cancer growth and metastasis in vivo. Our findings reveal that ZEB1-mediated upregulation of circWWC3 promotes breast cancer progression through activating Ras signaling pathway, which provides a potential therapeutic target and prognostic biomarker for breast cancer.

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Figure 1. ZEB1 Directly Upregulates Circular WWC3 (circWWC3) Expression

(A) Heatmap of hierarchical clustering indicates the top 15 upregulated (red) and downregulated (green) circRNAs. Two circRNAs (hsa_circ_0089866 and hsa_circ_0001910), which are derived from the same gene WWC3, were selected for further study. (B) The genomic structure of hsa_circ_0089866 and hsa_circ_0001910. The expression of hsa_circ_0001910 in MDA-MB-231 cells was detected by RT-PCR and then validated by Sanger sequencing. (C) Fluorescence in situ hybridization (FISH) demonstrated that circWWC3 preferentially localized in cytoplasm. (D) The expression of ZEB1, WWC3 circRNAs, mRNA, and pre-mRNA in ZEB1-overexpressed MDA-MB-231 cells was detected by qRT-PCR. *p < 0.05; not significant (NS), p > 0.05. (E) The putative ZEB1 binding motif in the WWC3 promoter was analyzed by JASPAR online database. (F) ChIP-PCR, using the specific antibody against ZEB1, was performed to validate whether ZEB1 could bind to the WWC3 promoter. *p < 0.05; NS, p > 0.05. (G) Dual-luciferase reporter assay was used to detect the effect of ZEB1 on the activity of the WWC3 promoter. *p < 0.05. (H) The luciferase reporter assay was performed in MDA-MB-231 cells cotransfected with wild-type or mutant WWC3 promoter luciferase constructs and ZEB1 overexpression vector. *p < 0.05; NS, p > 0.05.
breast cancer in vivo study revealed that short hairpin (sh)RNA-mediated knockdown of circWWC3 partially antagonized ZEB1-mediated breast cancer growth and metastasis. Our study revealed a novel function of the ZEB1/circWWC3 axis through activating the Ras signaling pathway in breast cancer progression and provided novel insights into underlying mechanism of breast cancer.

RESULTS

ZEB1 Directly Upregulates circWWC3 Expression
To investigate how circRNAs are regulated by ZEB1, we analyzed the circRNA expression profile after ZEB1 transfection using the Arrays-har human circRNA microarray in MDA-MB-231 cells. The circRNA expression profile was put into Tables S2 and S3. Based on the microarray results, we filtered out 15 upregulated and 15 downregulated circRNAs (Figure 1A). We noted that two circRNAs (hsa_circ_0089866 and hsa_circ_0001910), which are derived from the same gene WWC3, were upregulated after ZEB1 transfection (Figure 1A). The genomic structure shows hsa_circ_0089866 contains eight exons from exon 2 to exon 9 (1,068 nt), and hsa_circ_0001910 contains seven exons from exon 2 to exon 8 (825 nt) from the WWC3 gene flanked by long introns on either side (Figure 1B; Figures S1A and S1B). The distinct products of the expected sizes were amplified using outward-facing primers in breast cancer tissues and cell lines (Figures S1C and S1D). These two circular isoforms of WW C3 were resistant to RNase R, whereas the linear form RNA was significantly reduced after RNase R treatment (Figure S1E). Actinomycin D treatment revealed that the circRNA isoforms were highly stable, with significantly reduced after RNase R treatment (Figure S1E). The qRT-PCR results of the circRNA microarray, we examined the expression of circWWC3 using MDA-MB-231 cells cotransfected with wild-type or mutant WW C3 promoter luciferase construct and ZEB1 overexpression vector. The results indicated that the 2-kb promoter region (−1,255 bp to −1,255 bp upstream of the WW C3 transcription start site) is essential for ZEB1-mediated transcriptional activation of WW C3 gene (Figure 1H). Taken together, our results suggested as a transcription factor, ZEB1 increases circWWC3 expression through directly binding to WW C3 promoter.

circWWC3 Expression Is Associated with Breast Cancer Progression
To investigate the circWWC3 expression in breast cancer tissues and its effect on breast cancer malignancy, we randomly selected 156 cases of breast cancer tissues and examined the expression of circWWC3 using FISH (Figure 2A). As shown in Table S4, circWWC3 expression is positively associated with the clinical stage of breast cancer patients, suggesting that upregulation of circWWC3 indicates an aggressive characteristic of breast cancer. In addition, the survival analysis showed that elevated expression of circWWC3 indicated a poor overall survival of breast cancer patients (Figure 2B). These results demonstrated that circWWC3 may play an oncogenic role in breast cancer progression.

circWWC3 Increases Cell Proliferation, Migration, and Invasion of Breast Cancer Cells
To investigate the biological role of circWWC3 in breast cancer progression, we used RNA interference to silence the expression of circWWC3 in two circWWC3 high-expressed breast cancer cell lines. We designed two small interfering (si)RNAs to target the back-splice sequence of circWWC3 that did not affect the expression of the WW C3 linear species (Figure 2C). Subsequent functional experiments revealed that downregulation of circWWC3 significantly suppressed proliferation, migration, and invasion of MDA-MB-231 and MCF-7 cells. Interestingly, ZEB1 overexpression increased the WW C3 pre-mRNA level, but did not affect the expression of WW C3 mRNA (Figure 1D). To explore the transcription regulation of ZEB1 on circWWC3, we analyzed the promoter sequence of WW C3 and found a binding motif of ZEB1 on −1,255 bp to −1,255 bp upstream of the transcription start site (Figure 1E). Chromatin immunoprecipitation (ChiP)-PCR analysis revealed the occupancy of ZEB1 on −1,255 bp to −1,255 bp of the WW C3 promoter, suggesting ZEB1 directly binds to the WW C3 promoter (Figure 1F). The dual-luciferase reporter assay showed ZEB1 overexpression increased the WW C3 promoter activity, whereas ZEB1 knockdown decreased WW C3 promoter activity (Figure 1G). To further demonstrate whether WW C3 is a direct transcriptional target gene of ZEB1, the luciferase reporter assay was performed in MDA-MB-231 cells cotransfected with wild-type or mutant WW C3 promoter luciferase construct and ZEB1 overexpression vector. The results indicated that the 2-kb promoter region (−1,255 bp to −1,255 bp upstream of the WW C3 transcription start site) is essential for ZEB1-mediated transcriptional activation of WW C3 gene (Figure 1H). Taken together, our results suggested as a transcription factor, ZEB1 increases circWWC3 expression through directly binding to WW C3 promoter.
MCF-7 cells (Figures 2D–2K). These evidences implied that circWWC3 may function as a facilitating factor for breast cancer progression.

We also used RNA interference to knock down the expression of linear WW3C or circWW3C plus linear WW3C (Figure S3A) in MDA-MB-231 cells. Cell proliferation, migration, and invasion were not changed when both linear WW3C and circWW3C were knocked down. siRNA-mediated knockdown of linear WW3C increased proliferation, migration, and invasion of breast cancer cells (Figures S3B–S3E). Survival analysis from the Kaplan-Meier Plotter database showed that downregulation of linear WW3C expression was associated with the poor survival of breast cancer patients (Figure S3F). These data revealed that contrary to circWW3C, the linear form of WW3C plays a tumor-suppressive role in breast cancer progression. In addition, overexpression of circWW3C increased proliferation, migration, and invasion of MDA-MB-453 cells (Figures S4A–S4E). Taken together, our results demonstrated that circWW3C increases proliferation, migration, and invasion of breast cancer cells and plays oncogenic functions in breast cancer progression.

circWW3C Functions as the Sponge of miR-26b-3p and miR-660-3p

Because circWW3C has a high abundance in breast cancer and is located in the cytoplasm, we explored whether circWW3C binds to miRNAs as a miRNA sponge. Through analyzing the miRanda and TargetScan databases, the top 2 miRNAs were miR-26b-3p and miR-660-3p, based on the miRNA response element (MRE) analysis of putative binding sites in the circWW3C sequence (Figure 3A). We first detected the colocalization of circWW3C, miR-26b-3p, and miR-660-3p in MDA-MB-231 and MCF-7 cells. qRT-PCR and FISH results revealed that both circWW3C and miR-26b-3p or miR-660-3p were predominantly located in the cytoplasm (Figures 3B and 3C), which suggested that circWW3C has a condition to act as the sponge of miR-26b-3p and miR-660-3p. RNA-binding protein immunoprecipitation (RIP) results revealed that circWW3C could interact with miR-26b-3p and miR-660-3p in breast cancer MDA-MB-231 and MCF-7 cells (Figures 3D and 3E). The luciferase reporter assay showed that both miR-26b-3p and miR-660-3p inhibited the luciferase activity of luciferase (Luc)–circWW3C in both MDA-MB-231 and MCF-7 cells (Figure 3F). These data suggested that circWW3C may serve as a sponge for both miR-26b-3p and miR-660-3p.

circWW3C Maintained the Oncogenic Properties of Breast Cancer Cells through Acting as the Sponge of miR-26b-3p and miR-660-3p

To investigate the possible role of miR-26b-3p and miR-660-3p in breast cancer, we examined their expression using qRT-PCR and analyzed the effect of miR-26b-3p or miR-660-3p on the overall survival of breast cancer patients. Our results revealed that lower expression of miR-26b-3p and miR-660-3p indicated poor overall survival of breast cancer patients (Figure 4A). Our results were supported by the Kaplan-Meier Plotter database (Figure 4B). Functional experiments revealed that enforced expression of miR-26b-3p or miR-660-3p inhibited cell proliferation, migration, and invasion of MDA-MB-453 cells (Figures S5A–S5F). In miR-26b-3p or miR-660-3p high-expressed MDA-MB-231 and BT-549 cells, miR-26b-3p or miR-660-3p inhibitors increased cell proliferation, migration, and invasion (Figures S6A–S6H). Our results suggested that miR-26b-3p and miR-660-3p might play a tumor-suppressive role in breast cancer progression. The rescue experiment in MDA-MB-231 cells revealed that circWW3C suppression inhibited cell proliferation, migration, and invasion, whereas miR-26b-3p and miR-660-3p suppression rescued circWW3C knockdown-mediated inhibition of cell proliferation, migration, and invasion (Figures 4C–4F). Similar results were obtained in MCF-7 cells (Figures 4G–4I). These results suggested that high expression of circWW3C probably maintained the oncogenic properties of breast cancer cells through sponging miR-26b-3p and miR-660-3p. siRNA-mediated knockdown of circWW3C suppressed the oncogenic properties through releasing miR-26b-3p and miR-660-3p. siRNA-mediated knockdown of circWW3C might play a tumor-suppressive role in breast cancer.

Ras Signaling Pathway Is the Direct Pathway of miR-26b-3p and miR-660-3p in Breast Cancer Cells and Is Inhibited via circWW3C Knockdown

To gain further insight into the downstream pathways regulated by miR-26b-3p and miR-660-3p, we identified 2,234 putative cotarget genes of miR-26b-3p and miR-660-3p through the TargetScan database (Figure 5A). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that 45 pathways were associated with the cotarget genes of miR-26b-3p and miR-660-3p (Figure S7). In these pathways, “Ras signaling pathway” was most significantly associated with these target genes. Most of these genes are shown to have oncogenic potential in cancer progression, such as EGFR, GRB2, PAK4, MAPK1, and AKT1. Through the TargetScan database, multiple potential binding sites for miR-26b-3p and miR-660-3p seed regions on the 3’ UTR of these target genes were found (Figure S8). qRT-PCR and western blot results revealed that miR-26b-3p and miR-660-3p did not change the mRNA level (Figure 5B) but notably inhibited the protein expression of these genes in both MDA-MB-231 and MCF-7 cells (Figures 5C and 5D). Furthermore, we found that...
knockdown of circWWC3 also reduced the protein expression of these genes (Figures 5E and 5F). FISH and immunohistochemistry (IHC) results revealed that high expression of circWWC3 was associated with the high expression of these target genes (Figure 6A; Table S5). Kaplan-Meier analysis showed high expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 was associated with the poor overall survival of breast cancer patients (Figure 6B), which was supported by Kaplan-Meier Plotter database results (Figure 6C). Taken together, our results indicated that the Ras signaling pathway is the direct pathway of miR-26b-3p and miR-660-3p in breast cancer cells and is inhibited through circWWC3 knockdown.

**ZEB1 Promotes Breast Cancer Growth and Metastasis through Regulating the circWWC3-miR-26b-3p/miR-660-3p-Ras Signaling Pathway Axis In Vivo**

To investigate whether the ZEB1-circWWC3-miR-26b-3p/miR-660-3p-Ras signaling pathway axis affects breast cancer growth and metastasis, we applied the preclinical study using the in vivo mouse breast cancer xenograft and metastasis model with MDA-MB-231 cells expressing firefly luciferase. Female nude mice were randomly divided into four groups, as indicated in Materials and Methods. For the xenograft tumor model, an increase of the tumor luciferase signal in the ZEB1-infected group was found after 3 weeks (Figure 7A). According to the results, enforced expression of ZEB1 increased the tumor growth of breast cancer and enhanced the expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 (Figures 7B–7D). shRNA-mediated knockdown of circWWC3 could partly reverse the ZEB1-induced breast cancer growth and the expression of the above proteins (Figures 7B–7D). For the metastasis model, mice were sacrificed after 8 weeks, and the metastatic foci in lung and liver were observed by IVIS and counted by the naked eye (Figures 7E and 7F). The metastasis was further confirmed by H&E staining (Figure 7G). The results revealed that overexpression of ZEB1 increased the metastasis of breast cancer, whereas shRNA-mediated knockdown of circWWC3 partly reversed the ZEB1-induced breast cancer metastasis. Furthermore, ZEB1 expression was positively correlated with the expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 (Table S6). The survival analysis revealed that high expression of ZEB1 plus circWWC3 indicated a poor prognosis of breast cancer patients (Figure 7H). Taken together, these results proved that ZEB1 may play a promoting role on breast cancer growth and metastasis via modulation of the circWWC3-miR-26b-3p/miR-660-3p-Ras signaling pathway axis.

**DISCUSSION**

Accumulating evidence demonstrated that circRNAs play crucial roles in the development of breast cancer.21–25 circRNAs have been proven to be generated cotranscriptionally and compete with the canonical pre-mRNA splicing.16,26 As a zinc-finger transcription factor, ZEB1 has been widely recognized as an important driver of tumor growth and metastasis in cancer, especially in breast cancer.22 However, the role of ZEB1-regulated circRNAs in breast cancer progression still remains unexplored. In the present study, we examined the ZEB1-regulated transcription profile of circRNAs. As a result, we found that exogenous expression of ZEB1 induced the transcription of the circular form of WWC3 but not the linear WWC3. circWWC3 is highly abundant in breast cancer and was associated with a poor prognosis of breast cancer patients. Mechanically, circWWC3 promoted the tumor growth and metastasis of breast cancer through acting as the sponge of miR-26b-3p and miR-660-3p to target the Ras signaling pathway. Our results presented evidence that activation of ZEB1 and ZEB1-induced circWWC3 is important for breast cancer progression.

WWC3 has been identified as a tumor suppressor that is downregulated in several malignancies. Downregulation of WWC3 is associated with poor prognosis of cancer patients.28–30 WWC3 inhibits cell proliferation and metastasis through regulating the Hippo and Wnt signaling pathways.28–31 In our present study, linear WW3 was associated with good prognosis and inhibited breast cancer cell growth and metastasis. However, the circular form of WWC3 was dominant and exhibited oncogenic functions in breast cancer, indicating that the competition of circWWC3 with the linear WWC3 promoted the progression of breast cancer. Further studies are required to elucidate the mechanism of backsplicing formation of WWC3 pre-mRNA in breast cancer.

Due to the high abundance, stability, and potential number of MREs that they contain, some circRNAs could function as “miRNA sponges.”36,41,52 Due to the lack of free ends, circRNAs are resistant to exonucleases and more stable.

**Figure 4. circWWC3 Maintained the Oncogenic Properties of Breast Cancer Cells through Acting as the Sponge of miR-26b-3p and miR-660-3p**

(A) The lower expression of miR-26b-3p and miR-660-3p indicated poor overall survival of breast cancer patients in our study. (B) The Kaplan-Meier Plotter database (http://kmplot.com/analysis/) was used to validate our survival results. (C–F) circWWC3 suppression inhibited cell proliferation (C and D) and migration and invasion (E and F), whereas miR-26b-3p and miR-660-3p suppression rescued circWWC3 knockdown-mediated inhibition of proliferation, migration, and invasion of MDA-MB-231 cells. (G–J) circWWC3 suppression inhibited cell proliferation (G and H) and migration and invasion (I and J), whereas miR-26b-3p and miR-660-3p suppression rescued circWWC3 knockdown-mediated inhibition of proliferation, migration, and invasion of MCF-7 cells. *p < 0.05.
Figure 5. Ras Signaling Pathway Is the Direct Pathway of miR-26b-3p and miR-660-3p in Breast Cancer Cells and Is Inhibited via circWWC3 Knockdown
(A) 2,234 putative cotarget genes of miR-26b-3p and miR-660-3p were identified through the TargetScan database. (B) qRT-PCR was performed to detect the mRNA expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 after transfected miR-26b-3p and miR-660-3p mimics into MDA-MB-231 or MCF-7 cells. (C and D) Western blot was performed to detect the protein expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 after transfected miR-26b-3p and miR-660-3p mimics into MDA-MB-231 (C) or MCF-7 (D) cells. *p < 0.05. (E and F) Knockdown of circWWC3 reduced the protein expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 in MDA-MB-231 (E) and MCF-7 (F) cells. *p < 0.05.
than their linear isoforms. Moreover, some small circRNAs can be absorbed into exosomes. Therefore, circRNAs have the potential to function as the predicting markers for cancer diagnosis and prognosis.53–56 Therefore, the functions of circRNAs in cancer progression still need to be further explored.

In summary, our study can be concluded by the following major findings: (1) We identified a novel circRNA, circWWC3, that is induced by ZEB1 and promotes breast cancer growth and metastasis. (2) circWWC3 can act as the sponge of miR-26b-3p and miR-660-3p and inhibit their tumor-suppressive functions in breast cancer. (3)
The Ras signaling pathway is the target pathway of the circWWC3-miR-26b-3p/miR-660-3p axis in breast cancer. (4) ZEB1-mediated upregulation of circWWC3 promotes breast cancer progression through activating the Ras signaling pathway. Our study revealed that circWWC3 may function as an oncogene and act as a prognostic biomarker and therapeutic target for breast cancer.

MATERIALS AND METHODS

circRNA Microarray
Total RNA was extracted from MDA-MB-231 cells transfected with ZEB1 or empty vector. Arraystar Human Circular RNA Microarray v.2 (catalog number: AS-CR-H-V2.0; Arraystar, MD, USA) was used to identify differentially expressed circRNAs after transfection of ZEB1 in MDA-MB-231 cells. The sample preparation and microarray hybridization process were carried out based on Arraystar’s protocols.

Clinical Specimens
All human breast cancer tissues were collected from the Fourth Hospital of Hebei Medical University. All patients did not receive preoperative chemotherapy and radiation therapy. The human tissues were obtained with informed consent, and our study was approved by the Clinical Research Ethics Committee of our hospital.

Animal Experiment
The animal experiments were approved by Animal Care Committee of the Fourth Hospital of Hebei Medical University. For xenograft tumor experiment and in vivo metastasis experiment, 4-week-old female BALB/c nude mice were randomly divided into four groups (n = 5 for each group). MDA-MB-231-luciferase cells (1 × 10^6 cells per mouse) stably transfected with (1) empty vector or sh-scramble, (2) ZEB1 + sh-scramble, (3) empty vector + sh-circWWC3, or (4) ZEB1 + sh-circWWC3 were injected into mice subcutaneously for per mouse) stably transfected with (1) empty vector + sh-scramble, (3) empty vector + sh-circWWC3, or (4) ZEB1 + sh-circWWC3, or (4) ZEB1-mediated expression of ZEB1 plus circWWC3 indicated a poor prognosis survival of breast cancer patients.

In vivo
Figure 7. ZEB1 Promotes Breast Cancer Growth and Metastasis through Regulating the circWWC3-miR-26b-3p/miR-660-3p-Ras Signaling Pathway Axis

(A) An in vivo imaging system (IVIS) was performed to check the fluorescence signal of xenograft tumors. The representative bioluminescent images of three nude mice in each group were shown. (B) Tumor growth curve within 30 days was shown. *p < 0.05. (C and D) The expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 in mice tumor tissues was examined by IHC. (C): Representative images; (D): Quantitative analysis. (E and F) The metastatic foci in lung and liver were observed by IVIS and counted by the naked eye. *p < 0.05. (E): Representative images; (F): Quantitative analysis. (G) The metastasis was further confirmed by H&E staining. (H) The survival analysis revealed that high expression of ZEB1 plus circWWC3 indicated a poor prognosis survival of breast cancer patients.

SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
Study Concept and Design, L.M., C.G., and M.S.; Specimen Provider, X.F., L.G., and M.S.; Acquisition of Clinical Data, F.L., S.L., and Z.L.; Analysis and Interpretation of Data and Statistical Analysis, L.M., S.L., and M.S.; Animal Experiments, L.M. and Y.J.; Drafting of the Manuscript, L.M. and M.S.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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Supplemental Information

ZEB1-Mediated Transcriptional Upregulation of circWWC3 Promotes Breast Cancer Progression through Activating Ras Signaling Pathway

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Short Title: circWWC3 promotes breast cancer progression
Supplementary Materials and Methods

Cell culture, DNA/RNA extraction, RNA RNase R treatment

All cell lines were cultured in RPMI1640 (GIBCO, USA) added 10% fetal heat-inactivated bovine serum (GIBCO, USA) and 1% Penicillin-Streptomycin Solution (100×). Cells were grown in a 37°C, 5% CO₂ incubator. The genome DNA was extracted by TANGEN DNA extraction kit. Total RNA was extracted by TRIzol Reagent (Invitrogen, USA). RNase R treatment was performed by 3U/mg RNase R at 37°C for 15 min.

Plasmid construction

For circWWC3 overexpression plasmid, hsa_circ_0001910 (circBank ID: hsa_circWWC3_005) was cloned into pLC5-ciR vector and synthesized by Geneseed Biotech Co, Guangzhou, China. For circWWC3 luciferase reporter plasmid, the linear form of circWWC3 sequence was synthesized and cloned into psiCHECK™-2 vector (Geneseed Biotech Co, Guangzhou, China). For EGFR, GRB2, PAK4, MAPK1, AKT1 3’UTR plasmids, the 3’UTRs of these genes were synthesized and cloned into pGL3 vector (Promega, USA).

Reverse transcription-quantitative real-time PCR (qRT-PCR)

Total RNA preparation and qRT-PCR were performed as described in our previous study (34).

Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization was performed with probes specific for circWWC3, miR-26b-3p and miR-660-3p sequences. The Biotin-labeled circWWC3 probe and Digoxin-labeled miR-26b-3p or miR-660-3p probe were purchased from QIAGEN company (Germany). The probe sequences were shown in Supplementary table S1. For cell FISH, MDA-MB-231 and MCF-7 cells were seeded on coverslips and incubated overnight. The next day, cells were washed three times with PBS and fixed with 4% paraformaldehyde. Following prehybridization in PBS with 0.5% Triton X-100, the cells were hybridized with the above probes overnight at 37°C. The fluorescence signal of circWWC3 was detected by CyTM5-Streptavidin Conjugate (ZyMAXTM Grade, Invitrogen). The fluorescence signals of miR-26b-3p and miR-660-3p were
detected by Anti-Digoxigenin antibody (Abcam) and Tyramide SuperBoost™ Kit with Alexa Fluor™ 488 (Invitrogen). The nuclei were counterstained by DAPI and the images were taken under a ZEISS LSM 710 confocal microscope (Germany). For tissue FISH, deparaffinize the paraffin slides of breast cancer tissue in xylene and ethanol solutions firstly. The following prehybridization and hybridization processes were the same as above. Based on the cytoplasmic expression intensity of circWWC3, tumor tissue samples were classified as follows: negative or weak expression in most cells was defined as negative group, weak expression in most cells or moderate expression in <50% of cells was defined as low expression group, and moderate to strong expression in most cells was defined as high expression group.

**Subcellular fractionation**

Cytoplasmic and nuclear fractions were obtained from breast cancer cells using Nuclear/Cytosol Fractionation Kit (BioVision, Milpitas, CA, USA) according to the manufacturer’s protocol.

**Chromatin immunoprecipitation assay (ChIP)**

ChIP analysis was performed based on the standard protocol of Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (Millipore, USA). Immunoprecipitations were performed with 2µg ZEB1 antibodies or normal IgG. Primers used to measure the enrichment of ZEB1 promoter DNA sequence are presented in Supplementary Table S1. The enrichment fold of ChIP DNA was calculated as percentage of input. The PCR products were separated electrophoretically and visualized by ethidium bromide staining.

**Bioinformatics analysis**

The overall survival analysis of miR-26b-3p, miR-660-3p, linear WWC3, EGFR, GRB2, PAK4, MAPK1 and AKT1 was performed by Kaplan-Meier plotter database (http://kmplot.com/analysis/). KEGG pathway analysis of miR-26b-3p and miR-660-3p co-target genes was performed by Enrichr (http://amp.pharm.mssm.edu/Enrichr/).
HE staining and Immunohistochemistry (IHC)

HE staining and IHC were carried out as previously mentioned [34, 35]. Rabbit EGFR polyclonal antibody (proteintech), rabbit GRB2 polyclonal antibody (proteintech), rabbit PAK4 polyclonal antibody (proteintech), rabbit P38 MAPK (D13E1) XP monoclonal antibody (Cell Signaling), rabbit AKT monoclonal antibody (abcam), rabbit ZEB1 monoclonal antibody (Santa Cruz) were applied for IHC analysis. The immunoreactivity evaluation was operated by at least two experienced pathologists.

RNA-binding protein immunoprecipitation (RIP)

Myc-AGO2 vector was co-tranfected with either miR-26b-3p or miR-660-3p. 48 hours after transfection, immunoprecipitation for AGO2 antibody was performed. Briefly, cells were lysed by RIP wash buffer, added with RNase inhibitor and proteinase inhibitor. The cell lysate was mixed with normal human IgG or Myc antibody (Abcam, ab9106) coupled sepharose beads (Millipore, Germany) and rotated overnight at 4°C. Then, beads were washed five times with RIP washing buffer. RNA was extracted by TRIzol reagent. Purified RNA was subsequently applied to qRT-PCR to examine the enrichment folds of binding targets through respective primers.

Luciferase reporter assay

Cells were transfected with the indicated luciferase vectors. Cells were lysed with the lysis buffer, the luciferase activity was measured by the Dual-Luciferase Reporter Assay system (Promega, USA).

CCK-8 assay

Cells were seeded at a cell density of 5×10^3 cells/well in 96-well plates. After attachment overnight, CCK8 assays were performed at the indicated time periods. In brief, 10μl of CCK8 solution was added to each well. After one hour of incubation at 37 °C, the absorbance readings for each well were carried out at 450nm using the microplate reader (Tecan, USA).

Colony formation assay

Cells with the indicated conditions were seeded into 6-well plates. The cells
were maintained at 37 °C for 10 days. Cell colonies were fixed and stained with Giemsa. Colonies were observed and calculated under a microscope.

**Wound healing assay**

Cells with the indicated conditions were grown to 80% confluence. Then, the pipette tips were utilized to scratch the monolayer cells. After 24 hours, the migration distance across the wound injury was observed under the microscope. The 0h cell images were normalized as the control to calculate a value of relative migration rate.

**Transwell migration and matrigel invasion assays**

The migration and matrigel invasion assay was performed by transwell empty chamber (for migration assay) or pre-coated matrigel chamber (for invasion assay) based on the manufacturer’s standard protocol (BD corporation, USA). The cell suspensions of different groups were added to the upper chambers in a density of 5×10^4 cells/well and incubated for 24-48h. The migration and invasion cell numbers were quantified by five random fields under the microscope.

**Western blot**

Western blot was performed based on the standard protocol (34). Rabbit EGFR polyclonal antibody (proteintech), rabbit GRB2 polyclonal antibody (proteintech), rabbit PAK4 polyclonal antibody (proteintech), rabbit P38 MAPK (D13E1) XP monoclonal antibody (Cell Signaling), rabbit AKT monoclonal antibody (abcam), rabbit β-actin (proteintech) were used for the immunoreactivity. The expression levels were visualized under enhanced chemiluminescence.
Supplementary Figures

Supplementary Figure S1. Identification of circWWC3 in breast cancer cells and tissues. A and B, The genomic structure of hsa_circ_0089866 and hsa_circ_0001910. C and D, The PCR products of the expected sizes were amplified using outward-facing primers in breast cancer tissues and cell lines. E, qRT-PCR revealed that these two circular isoforms of WWC3 was resistant to RNase R, whereas the linear form RNA was significantly reduced after RNase R treatment. *P<0.05, NS: P>0.05. F, qRT-PCR analysis of these two circular isoforms of WWC3 (hsa_circ_0089866 and hsa_circ_0001910) and WWC3 mRNA in Actinomycin D-treated MDA-MB-231 cells.
Supplementary Figure S2. The expression abundance of circWWC3 in breast cancer cells and tissues. A and B, The qRT-PCR results using outward-facing primers showed that hsa_circ_0001910 has a higher abundance than hsa_circ_0089866 in breast cancer cells and tissues. C, Sequencing data in ~ 1000 human cancer cell lines also showed that hsa_circ_0001910 is highly expressed in breast cancer (BRCA) lineage.
Supplementary Figure S3. Cell function changes after knocking down the expression of linear WWC3 or circWWC3 plus linear WWC3. A, RNA interference was used to knock down the expression of linear WWC3 or circWWC3 plus linear WWC3 in MDA-MB-231 cells. *P<0.05. B and C, CCK8 and colony formation assay were performed to detect the proliferation and colony-forming ability of MDA-MB-231 in linear WWC3 or circWWC3 plus linear WWC3 knocked-down group. *P<0.05. D and E, Wound healing experiment and transwell assay were performed to detect the migration and invasion ability of MDA-MB-231 cells in linear WWC3 or circWWC3 plus linear WWC3 knocked-down group. *P<0.05. F, Survival analysis from Kaplan-Meier plotter database (http://kmplot.com/analysis/) showed that down-regulation of linear WWC3 expression was associated with the poor survival of breast cancer patients.
Supplementary Figure S4. Overexpression of circWWC3 increased breast cancer cell proliferation, migration and invasion. A, qRT-PCR was used to detect the expression of circWWC3 and linear WWC3 after overexpression of circWWC3 in MDA-MB-453 cells. *P<0.05. B and C, CCK8 and colony formation assay were performed to detect the proliferation and colony-forming ability of MDA-MB-453 cells after transfection of circWWC3. *P<0.05. D and E, Wound healing experiment and transwell assay were performed to detect the migration and invasion ability of MDA-MB-453 cells after overexpression of circWWC3. *P<0.05.
**Supplementary Figure S5.** miR-26b-3p or miR-660-3p mimics inhibited breast cancer cell proliferation, migration and invasion. A, qRT-PCR was used.
to detect the expression of miR-26b-3p and miR-660-3p in breast cancer cell lines. B, The expression of miR-26b-3p and miR-660-3p in MDA-MB-453 cells transfected with miR-26b-3p or miR-660-3p mimics was detected by qRT-PCR. *P<0.05. C and D, Enforced expression of miR-26b-3p or miR-660-3p inhibited proliferation and colony-forming ability of MDA-MB-453 cells. *P<0.05. E and F, Enforced expression of miR-26b-3p or miR-660-3p inhibited migration and invasion of MDA-MB-453 cells. *P<0.05.
Supplementary Figure S6. miR-26b-3p or miR-660-3p inhibitor increased breast cancer cell proliferation, migration and invasion. A and E, CCK8 assay revealed that miR-26b-3p or miR-660-3p inhibitor increased proliferation of MDA-MB-231 and BT-549 cells. *P<0.05. B and F, Colony formation assay showed that miR-26b-3p or miR-660-3p inhibitor increased colony forming ability of MDA-MB-231 and BT-549 cells. *P<0.05. C and G, Wound healing experiment showed that miR-26b-3p or miR-660-3p inhibitor increased migration of MDA-MB-231 and BT-549 cells. *P<0.05. D and H, Transwell migration and matrigel invasion assay revealed that miR-26b-3p or
miR-660-3p inhibitor increased migration and invasion of MDA-MB-231 and BT-549 cells. *P<0.05.

Supplementary Figure S7. KEGG pathway analysis revealed that 45 pathways were associated with miR-26b-3p and miR-660-3p co-target genes.
Supplementary Figure S8. TargetScan database analysis showed multiple potential binding sites of miR-26b-3p (A) and miR-660-3p (B) seeds regions on the 3'UTRs of their co-target genes.

| Position | 4035-4032 of EGFR 3' UTR | 5'...GUUCAUGCUUACUUACAGAACAGA... | miR-26b-3p | 3'...CUUGUUAUCAUCUUACCUUGCC |
|----------|---------------------------|------------------------------------|------------|-----------------------------|
|           | 14|                             | Supplementary Figure S8. TargetScan database analysis showed multiple potential binding sites of miR-26b-3p (A) and miR-660-3p (B) seeds regions on the 3'UTRs of their co-target genes. |
**Supplementary Tables**

**Supplementary Table S1 Sequences of primers and probes**

### Primer sequences

| Gene          | Primer sequence                  |
|---------------|----------------------------------|
| hsa_circ_0001910 | **forward**, 5'-CTTCAAGAAGACAGCTGCTCCG-3'  |
|               | **reverse**, 5'-CCAACACAAATCGGCAAAGGT-3'  |
| hsa_circ_0089866 | **forward**, 5'-TCAGCTCATCATTGCACAGC-3'  |
|               | **reverse**, 5'-CCAACACAAATCGGCAAAGGT-3'  |
| WWC3-linear mRNA | **forward**, 5'-TAGCAAGTGCTCGGATAGG-3'  |
|               | **reverse**, 5'-CGCTCTATCCAGTTTGAGC-3'  |
| WWC3 pre-mRNA | **forward**, 5'-TTCGACAGCCACATACCCTCTATCTTG-3'  |
|               | **reverse**, 5'-CTAAAGTGAAAGATGCCGGGCACAAAAGG-3'  |
| CHIP-Primer 1 | **forward**, 5'-TCACGAGATCAGACCCACA-3'  |
|               | **reverse**, 5'-GCGAAGGTGGGAAAAGGC-3'  |
| CHIP-Primer 2 | **forward**, 5'-GGAGGGAGAGAGCGAGGA-3'  |
|               | **reverse**, 5'-CTCTGGCTTTTTCACCACCAC-3'  |
| GAPDH         | **forward**, 5'-AGCCACATCGCTAGACAC-3'  |
|               | **reverse**, 5'-GCCCAATACGACCAAATCC-3'  |
| EGFR          | **forward**, 5'-AGGTGAAAAACAGCTGAAGG-3'  |
|               | **reverse**, 5'-AGGTGATGTGTCAGGCTGA-3'  |
| GRB2          | **forward**, 5'-AGACGGCCTTCATTCCAAGA-3'  |
|               | **reverse**, 5'-TGCTGCACATCGTTTCAA-3'  |
| PAK4          | **forward**, 5'-TGAGTGTGCACTGTGTGTGG-3'  |
|               | **reverse**, 5'-CTGCTGCCAGTGCACTGCA-3'  |
| MAPK1         | **forward**, 5'-GTAGAGTGGTTAGGGCTTCTT-3'  |
|               | **reverse**, 5'-TGAGATGTCGGGGCTTCTTT-3'  |
| AKT1          | **forward**, 5'-CGACGTGGCTATGTGAAGG-3'  |
|               | **reverse**, 5'-GATGATGAGGGTGTGGGCC-3'  |

### Probe sequences

| Gene          | Probe sequence                  |
|---------------|---------------------------------|
| hsa-circ-0001910 | /5'BiosG/TCAATGGCCTTTTGTATCTCTCCTTCT/3'Bio/  |
| hsa-miR-26b-3p | /5'DIG_N/AGCCAAAGTAATGGGAA/3'DIG_N/  |
siRNA sequences
si-hsa_circ_0001910_001    5’-CCAGAAAGAGGATAACAAA-3’
si-hsa_circ_0001910_002    5’-GAAAGAGGATAACAAAGCC-3’
si-hsa_circ_0001910_003    5’-AGAGGATAACAAAGCCATT-3’
si-linear-human-WWC3_001   5’-GACCAAACCTTGACTACCGT-3’
si-linear-human-WWC3_002   5’-GGCAACAAACAAACCATCCA-3’
si-linear-human-WWC3_003   5’-GCCATGACAAGGAAAGAAA-3’
si-both-human-WWC3_001    5’-GGACTTCCCTCACCATGTA-3’
si-both-human-WWC3_002    5’-GTAGACCGAGTCAGACTTA-3’
si-both-human-WWC3_003    5’-TGACCCACACCAATTAAA-3’

Supplementary Table S2 and Supplementary Table S3 have been uploaded as Excel files under the "Supplemental Videos and Spreadsheet" category.

Supplementary Table S4 The association between circWWC3 expression and the clinicopathological characteristics of breast cancer patients

| Parameters           | Groups | Cases | circWWC3 | X² | P   |
|----------------------|--------|-------|----------|----|-----|
|                      |        |       | Low      | High |     |
| Age (yrs)            | ≥50    | 96    | 54       | 42  | 0.127 | 0.722 |
|                      | <50    | 60    | 32       | 28  |      |      |
| Tumor size           | ≥5cm   | 142   | 77       | 65  | 0.521 | 0.470 |
|                      | <5cm   | 14    | 9        | 5   |      |      |
| Grade                | G1-2   | 75    | 39       | 36  | 0.571 | 0.450 |
|                      | G3     | 81    | 47       | 34  |      |      |
| Clinical stage       | II     | 110   | 62       | 48  | 7.621 | 0.022 |
|                      | III    | 36    | 15       | 21  |      |      |
| Molecular subtypes   | Luminal| 102   | 59       | 43  | 2.550 | 0.279 |
|                      | HER2+  | 25    | 11       | 14  |      |      |
|                      | Basal like | 29 | 13 | 16 |      |      |
Supplementary Table S5 The association between circWWC3 expression and the expression of genes on Ras signaling pathway

| Parameters | Groups | Cases | circWWC3 | $X^2$ | P    |
|------------|--------|-------|----------|-------|------|
|            |        |       | Low      | High  |      |      |
| ZEB1       | Low    | 84    | 71       | 13    | 63.575 | 0.000 |
|            | High   | 72    | 15       | 57    |       |      |
| EGFR       | Low    | 72    | 66       | 6     | 72.166 | 0.000 |
|            | High   | 84    | 20       | 64    |       |      |
| GRB2       | Low    | 72    | 57       | 15    | 31.235 | 0.000 |
|            | High   | 84    | 29       | 55    |       |      |
| PAK4       | Low    | 87    | 55       | 32    | 5.204  | 0.023 |
|            | High   | 69    | 31       | 38    |       |      |
| MAPK1      | Low    | 85    | 59       | 26    | 15.403 | 0.000 |
|            | High   | 71    | 27       | 44    |       |      |
| AKT1       | Low    | 92    | 63       | 29    | 16.157 | 0.000 |
|            | High   | 64    | 23       | 41    |       |      |

Supplementary Table S6 The association between ZEB1 expression and the expression of genes on Ras signaling pathway

| Parameters | Groups | Cases | ZEB1 | $X^2$ | P    |
|------------|--------|-------|------|-------|------|
|            |        |       |      |       |      |
| EGFR       | Low    | 72    | 66   | 6     | 76.961 | 0.000 |
|            | High   | 84    | 18   | 66    |       |      |
| GRB2       | Low    | 72    | 59   | 13    | 42.479 | 0.000 |
|            | High   | 84    | 25   | 59    |       |      |
| PAK4       | Low    | 87    | 62   | 25    | 24.013 | 0.000 |
|            | High   | 69    | 22   | 47    |       |      |
| MAPK1      | Low    | 85    | 64   | 21    | 34.570 | 0.000 |
|            | High   | 71    | 20   | 51    |       |      |
| AKT1       | Low    | 92    | 68   | 24    | 36.335 | 0.000 |
|            | High   | 64    | 16   | 48    |       |      |