circ-NOL10 regulated by MTDH/CASC3 inhibits breast cancer progression and metastasis via multiple miRNAs and PDCD4

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Circular RNAs (circRNAs) play important roles in carcinogenesis. Here, we investigated the mechanisms and clinical significance of circ-NOL10, a highly repressed circRNA in breast cancer. Subsequently, we also identified RNA-binding proteins (RBPs) that regulate circ-NOL10. Bioinformatics analysis was utilized to predict regulatory RBPs as well as circ-NOL10 downstream microRNAs (miRNAs) and mRNA targets. RNA immunoprecipitation, luciferase assay, fluorescence in situ hybridization, cell proliferation, wound healing, Matrigel invasion, cell apoptosis assays, and a xenograft model were used to investigate the function and mechanisms of circ-NOL10 in vitro and in vivo. The clinical value of circ-NOL10 was evaluated in a large cohort of breast cancer by quantitative real-time PCR. Circ-NOL10 is downregulated in breast cancer and associated with aggressive characteristics and shorter survival time. Upregulation of circ-NOL10 promotes apoptosis, decreases proliferation, and inhibits invasion and migration. Furthermore, circ-NOL10 binds multiple miRNAs to alleviate carcinogenesis by regulating PDCD4. CASC3 and metadherin (MTDH) can bind directly to circ-NOL10 with characterized motifs. Accordingly, ectopic expression or depletion of CASC3 or MTDH leads to circ-NOL10 expression changes, suggesting that these two RBPs modulate circ-NOL10 in cancer cells. circ-NOL10 is a novel biomarker for diagnosis and prognosis in breast cancer. These results highlight the importance of therapeutic targeting of the RBP-noncoding RNA (ncRNA) regulation network.

INTRODUCTION

Breast cancer is the most common and heterogeneous disease among women in the world.1 Despite continuous development of early diagnosis and improvement of treatments over the past few decades, there are still limited efficient therapies, especially in subtypes such as triple-negative breast cancer (TNBC). The median overall survival is only 10–13 months for metastatic TNBC.1 Therefore, understanding the molecular pathogenesis involved in cancer development and identifying novel biomarkers are essential to determine diagnosis, treatment strategies, and prognosis. Over the last few years, accumulating reports have found that noncoding RNAs (ncRNAs), including circular RNAs (circRNAs), are implicated widely in breast cancer progression.3,6 circRNA is a circular form of endogenous ncRNA produced mainly by circularization of specific exons. Expression analyses have indicated that it is tissue-specific or developmental stage-specific and evolutionarily conserved between mice and humans.5,6 A variety of molecular mechanisms have been proposed to explain circRNA function in human cancer, including acting as microRNA (miRNA) decoys, regulating gene splicing or transcription, translating peptides, and epigenetic regulation.4,7,8 Further, circRNAs are stable in formalin-fixed, paraffin-embedded tissue and human biofluids, such as exosomes, saliva, and plasma, indicating their potential diagnostic values9–11.

In breast cancer, we and others have discovered that several circRNAs inhibit tumor progression or promote tumorigenesis.12–14 For example, circEPSTI1 has been found to be increased significantly in TNBC samples. This circRNA is also a prognostic marker.15 Using a high-throughput circRNA microarray profiling platform, we identified a set of significantly decreased circRNAs in a large cohort of individuals with breast cancer. We elucidated the functions and molecular mechanisms of circTADA2As in breast cancer. Among these differentially expressed circRNAs, circ-NOL10 was the most downregulated in TNBC samples. In this study, we first investigated the clinical significance of circ-NOL10. Subsequently, we explored its biological roles and molecular mechanism during breast cancer progression. Furthermore, our bioinformatics analysis predicted that two RBPs (metadherin...
[MTDH] and CASC3 exon junction complex subunit [CASC3]) can directly bind to circ-NOL10, which is confirmed by follow-up experiments. Overall, we identified a novel RBP-ncRNA signaling route formed by two RBPs (MTDH and CASC3), one circRNA (circ-NOL10), three miRNAs (miR-149-5p, miR-330-3p, and miR-452-5p), and the effector PDCD4 in breast cancer carcinogenesis.

RESULTS

Circ-NOL10 is downregulated in breast cancer and associated with aggressive characteristics

We performed qPCR analyses on 178 breast cancer tissues (LA, N = 25; LB, N = 21; Her-2, N = 17; TNBC, N = 115) and 16 normal mammary gland tissues. Expression of circ-NOL10 is significantly lower in breast cancer tissue, which is consistent with our previous finding by circRNA array.13 The reduction of circ-NOL10 is more evident in LA and TNBC tissues (Figure 1A). Correspondingly, the expression levels of circ-NOL10 in 10 breast cancer cell lines were lower than in the immortalized mammary gland cell line MCF-10A (Figure 1B).

Notably, decreased circ-NOL10 expression is correlated significantly with advanced clinical stage (p = 0.018), increased lymphatic metastasis (p = 0.032), recurrence (p = 0.001), and death (p = 0.001) in individuals with TNBC (Table 1). These results suggest an association between downregulation of circ-NOL10 and aggressive characteristics of TNBC. Receiver operating characteristic (ROC) curves for circ-NOL10 expression were calculated. The area under the curve (AUC) for TNBC was 0.9212 (p < 0.0001; Figure 1F). Additionally, the AUCs for LA, LB, and Her-2 were 0.9275, 0.7619, and 0.9154, respectively (Figures 1C–1E), indicating that circ-NOL10 might be a promising diagnostic indicator for breast cancer. Next, according to the cutoff value of the ROC curve, individuals with TNBC were divided into two groups for disease-free survival (DFS) and overall survival (OS) analyses. Interestingly, low circ-NOL10 expression was significantly related to shorter DFS (p = 0.032) and OS (p = 0.027) (Figures 1G and 1H). Univariate Cox proportional hazards regression analyses indicated that circ-NOL10, like clinical stage, T classification status, and lymphatic metastasis, is a risk factor for DFS of individuals with TNBC (HR = 4.616, p = 0.043) and OS (HR = 3.886, p = 0.045) but not an independent prognostic factor for poor DFS and OS in the multivariate Cox model (Tables S1 and S2).

Circ-NOL10 inhibits breast cancer progression and metastasis

In view of circ-NOL10 being downregulated in breast tissue and cell lines, we overexpressed circ-NOL10 to further study its potential function in the MDA-MB-231 and MCF-7 cell lines. As shown in Figure 2A, ectopic expression of circ-NOL10 was increased significantly, indicating good overexpression efficiency. Upregulation of circ-NOL10 decreased cell proliferation (Figure 2B) and inhibited colony formation and invasion (Figures 2C–2D). Moreover, the wound healing assay demonstrated that ectopic expression of circ-NOL10 significantly inhibited migration of MDA-MB-231 and MCF-7 cells (Figure 2E). In addition, we investigated whether circ-NOL10 affects cell apoptosis using a flow cytometry assay. The results showed that early apoptosis could be triggered by circ-NOL10 overexpression but late apoptosis could not (Figure 2F).

To further confirm the function of circ-NOL10, we designed two small interfering RNA (siRNA)-circ-NOL10 products.
Tumor growth in the xenograft tumor model verified that circ-NOL10 can inhibit BC tumor growth in vivo.

Circ-NOL10 interacts with and sequesters miR-149-5p, miR-330-3p, and miR-452-5p

To explore the molecular mechanism of circ-NOL10 in breast cancer, we determined the subcellular location of circ-NOL10 in MDA-MB-231 and MCF-7 cells with fluorescence in situ hybridization (FISH). As shown in Figure 5A, circ-NOL10 was expressed in the cytoplasm and nucleus in cancer cells. Previous studies have demonstrated that some circRNAs can function as miRNA sponges in breast cancer,13,15 so we combined several bioinformatics tools to predict miRNAs that potentially bind to circ-NOL10 (Figure 4B). Furthermore, we used the mirgator.kobic3.0 database to select eight miRNAs that are expressed abundantly in breast cancer tissues and have binding sites on circ-NOL10 (Figure 4C). Next, miR-149-5p, miR-330-3p, miR-452-5p, and miR-767-5p were selected manually for experimental verification. Dual-luciferase reporter assays were performed with a recombinant reporter plasmid containing a luciferase gene and the circ-NOL10 sequence (psiCHECK2-circ-NOL10). A schematic of psiCHECK2-circ-NOL10 and circ-NOL10 recognition sites is shown in Figure 5D. Co-transfected psiCHECK2-circ-NOL10 and miR-149-5p, miR-330-3p, and miR-452-5p significantly decreased firefly luciferase reporter activity, but there was no significant change for co-transfected psiCHECK2-NOL10 and miR-767-5p (Figure 5E). These results indicate that miR-149-5p, miR-330-3p, and miR-452-5p can bind to circ-NOL10. Furthermore, the relative expression of miR-149-5p, miR-330-3p, and miR-452-5p were reduced significantly after overexpression of circ-NOL10 in breast cancer cells (Figure 5F). Finally, we checked cellular apoptosis after co-transfection of these miRNAs or circ-NOL10 alone in cells. We found that overexpression of these three miRNAs in the circ-NOL10-treated group can significantly decrease cellular apoptosis in MDA-MB-231 and MCF-7 cells (Figures 5G and 5H). These results confirmed our hypothesis that circ-NOL10 can interact with and sequester miR-149-5p, miR-330-3p, and miR-452-5p in breast cancer.

Circ-NOL10 suppresses carcinogenesis by regulating PDCD4

Next we combined five bioinformatic databases (TargetScan, miRNAorg, PITA, PicTar, and miRDB) to analyze the potential miRNA targets. Four genes (TUSC2, PDCD4, GRIA3, and CCBE1) were selected based on overlap of these prediction results for follow-up experimental verification (Figure S2A). After ectopic expression of circ-NOL10, we found that only PDCD4 was increased significantly compared with vector transfection at the protein level (Figures S2B and S2C). Because miRNAs generally regulate target gene expression by inhibiting translation, we decided to focus on PDCD4 in the following experiments. As a tumor suppressor with multi-functions, PDCD4 has been reported to be involved in proliferation, migration, invasion, and apoptosis.16–18 Downregulation of PDCD4 was also associated with poor prognosis in previous studies.19,20 Importantly, it is a known target gene for miR-330-3p in esophageal cancer.21 After transfection of the circ-NOL10 plasmid or circ-NOL10 siRNAs, the protein level of PDCD4 was increased accordingly in circ-NOL10-overexpressing cells and decreased in siRNA circ-NOL10-treated cells (Figure 6A). Then we used two different siRNAs to inhibit expression of PDCD4 (Figure 6B). As shown in Figures 6C–6G, cell invasion,
migration, and apoptosis assays indicated that knockdown of PDCD4 partially abolishes the effects of circ-NOL10 on breast cancer cells. Additionally, western blotting was performed to examine whether PDCD4 protein expression was affected by miR-149-5p, miR-330-3p, and miR-452-3p. In MDA-MB-231 cells, a fluorescence-activated cell sorting (FACS)-based assay indicated that apoptosis was
Figure 3. Knockdown of circ-NOL10 promotes breast cancer progression

Experiments were conducted after transfecting MCF-7 and MDA-MB-231 cells with circ-NOL10 siRNAs for 24 h. (A) circ-NOL10 expression after transfecting was analyzed by qPCR. (B) The effect of circ-NOL10 siRNAs on cell viability was analyzed by CCK-8. (C) The effect of circ-NOL10 siRNAs on colony formation was determined via a clonogenicity assay (top, representative pictures; bottom, quantitative bar for colony numbers). (D) The effect of circ-NOL10 siRNAs on cell invasion was detected via a Transwell assay (left, morphological comparison of cell penetration; right, quantitative bar for number of penetrating cells). (E) The effect of circ-NOL10 siRNAs on cell migration was measured by a wound scratch assay (left, representative images; right, quantitative bar for cell migration rate). (F) The effect of circ-NOL10 siRNAs on early apoptosis was analyzed by flow cytometry in cells (left, representative images; right, quantitative bar for cell apoptosis rate). Error bars represent the mean ± SEM from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
promoted following transfection of miRNA inhibitors (Figure S2D). Accordingly, protein expression of PDCD4 was upregulated significantly. Apoptosis-related markers, cleaved caspase-3 and Bad, were also increased significantly after downregulation of miR-149-5p, miR330-3p, and miR452-5p (Figures S2E and S2F). Upregulation of circ-NOL10 induced PDCD4 and apoptosis marker expression, and the effects could be reversed by miR-149-5p, miR-330-3p, and miR-452-5p mimics (Figures S2G and S2H). These data indicate that circ-NOL10 exerts a biological function via a circ-NOL10/miR-149-5p/miR-330-3p/miR-452-5p/PDCD4 pathway.

**MTDH and CASC3 regulate formation of circ-NOL10 in breast cancer**

An essential next step for understanding circRNA function is to identify key determinants in circRNA biogenesis. We reasoned that if a gene is a bona fide regulator, then it should have following characteristics: (1) alterations such as point mutation, copy number variation, and aberrant mRNA expression of this gene have been found in breast cancer, and (2) these alterations may have led to pathogenic changes and are correlated with survival. Based on these ideas, we analyzed multi-omics data of 825 BC samples and predicted 25 potential regulatory RBPs (materials and methods; Table S3). ADAR and FUS are involved in some circRNAs’ life cycle.22,23 Thus, combining our bioinformatics analysis and previous reports, we selected seven RBPs (ADAR, FUS, SRSF1, CWC15, CASC3, MTDH, and ESRP1) for further experiments. We constructed seven RBPs’ overexpression plasmids to examine whether they controlled aberrant expression of breast cancer-associated circ-NOL10. First, qPCR analyses showed that expression of circ-NOL10 was downregulated in cells’ ectopic expression of CASC3 or MTDH, but cells treated with the other five RBPs did not show a consistent pattern (Figure S3A). OS analyses found that individuals with more alterations on these two RBPs had a poor prognosis in the BRCA dataset, suggesting that they play crucial roles in carcinogenesis (Figure S3B). A siRNA assay was used to further verify the association of MTDH and CASC3 with circ-NOL10 (Figures 7A and 7B). The qPCR results indicated that inhibition of MTDH and CASC3 resulted in a significant increase of circ-NOL10 in MDA-MB-231 and MCF-7 cells (Figure 7C). These findings confirmed that CASC3 and MTDH could disrupt circ-NOL10 expression.

An RNA immunoprecipitation (RIP) assay was performed to determine whether MTDH and CASC3 can bind directly to circ-NOL10. Immunofluorescence and FISH (IF-FISH) analysis confirmed co-localization of circ-NOL10 with MTDH and CASC3 in the cytoplasm of MDA-MB-231 (Figure 7D). We transfected the pEZ-93-MTDH and pEZ-93-CASC3 plasmids, which express the encoded proteins fused to a FLAG tag, and individually overexpressed the two RBPs in MDA-MB-231 cells (Figure 7E). Bound complexes were pulled down using an antibody against FLAG. The qPCR results indicated that circ-NOL10 was highly enriched in the FLAG-containing group compared with the immunoglobulin G (IgG) isotype control (Figure 7F). We further verified the interactions between circ-NOL10 and endogenous RBPs by using a specific antibody against CASC3 and MTDH (Figure 7G).

To determine the binding motifs, the immunoprecipitated RNAs were also sequenced on an Illumina NovaSeq 6000 system. Bioinformatics analyses of RIP-seq reads showed two putative binding sites of MTDH and one of CASC3 on the circ-NOL10 (Figure 7H). As shown in Figure 7I, the luciferase activity of the reporter with the wild-type circ-NOL10 sequence (Luc-circ-NOL10) was inhibited significantly by MTDH and CASC3. This decrease was attenuated by mutation of MTDH binding sites (Luc-circ-N-MUT A and Luc-circ-N-MUT B) or the CASC3 binding site (Luc-circ-N-MUT C). These experimental results suggest that CASC3 and MTDH bind circ-NOL10 to regulate its formation in breast cancer. Importantly, these results also demonstrate that our bioinformatics predictions can be verified experimentally.

**DISCUSSION**

Our results demonstrated that circ-NOL10 inhibits breast cancer cell progression. Mechanistic studies indicated that MTDH and CASC3 converge at downregulating circ-NOL10 to release a set of three miRNAs. Competitive binding with the three miRNAs, in turn, blocks expression of the target gene PDCD4. Then decreased PDCD4 inhibits apoptosis and promotes cell proliferation, migration, and invasion (Figure 8). Thus, circ-NOL10 plays a central role in linking the upstream stimuli with downstream effectors in the RBP-ncRNA regulation network.

Accumulated evidence has shown correlations between circRNA expression and clinicopathological parameters such as tumor size, OS time, and tumor staging in various cancers.12,24,25 Notably, our
Figure 5. Circ-NOL10 interacts with and sequesters miR-149-5p, miR-330-3p, and miR-452-5p in breast cancer cells

(A) The distribution of circ-NOL10 in MDA-MB-231 and MCF-7 cells detected by FISH. Red, Cy3-labeled probes specific to circ-NOL10; green, FITC-labeled probes specific to 18S RNA; blue, DAPI stain for nuclei; merge represents an overlay figure. Scale bar, 20 μm. (B) Multiple bioinformatics tools were used to find miRNA and circ-NOL10 interaction. (C) An illustration showing the putative binding sites of circ-NOL10 with miRNAs. miR-452-5p has two binding sites. (D) A schematic of the psiCHECK2-circ-NOL10 vector and circ-NOL10 recognition sites. (E) Luciferase activity of psiCHECK2-circ-NOL10 vector and circ-NOL10 recognition sites. (F) miRNA expression after transfection of the circ-NOL10 overexpression plasmid or a control vector in MDA-MB-231 cells (left panel) and in MCF-7 cells (right panel). (G and H) The effect of circ-NOL10 on early apoptosis after transfection of circ-NOL10 or co-transfection with miR-149-5p, miR-330-3p, or miR-452-5p in MDA-MB-231 and MCF-7 cells (left, representative images; quantitative bar for cell apoptosis rate). *, circ-NOL10+miR NC compared with vector+miR NC; #, circ-NOL10+miRNA compared with circ-NOL10+miR NC. *, #p < 0.05; **, ##p < 0.01; ###p < 0.001.
Figure 6. Circ-NOL10 suppresses breast cancer progression by regulating PDCD4

(A) Western blot of PDCD4 after transfection of the overexpression circ-NOL10 plasmid or siRNAs in cells. (B) Expression of PDCD4 mRNA was examined after transfection two different siRNAs in cells. (C) Cell viability was analyzed by CCK-8. (D) Colony formation was determined via a clonogenicity assay (left, representative pictures; right, quantitative bar for colony numbers). (E) Cell invasion was detected via a Transwell assay (left, morphological comparison of cell penetration; right, quantitative bar for number of penetrating cells). (F) Cell migration was measured by a wound scratch assay (left, representative images; right, quantitative bar for cell migration rate). (G) Early cell apoptosis was analyzed by flow cytometry in cells (left, representative images; right, quantitative bar for cell apoptosis rate). Error bars represent the mean ± SEM from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 7. MTDH and CASC3 regulate formation of circ-NOL10

(A–C) After transfecting cells for 48 h with MTDH and CASC3 siRNA#1 and siRNA#2, (A) MTDH and CASC3 mRNAs were analyzed by qRT-qPCR, (B) MTDH and CASC3 proteins were analyzed by western blot, and (C) circ-NOL10 expression was analyzed by qPCR. (D) IF-FISH assay showing that circ-NOL10 colocalized with MTDH and CASC3 proteins in MDA-MB-231 cell cytoplasm. Red, Cy3-labeled probes specific to circ-NOL10; green, MTDH and CASC3 protein; blue, DAPI stain for nuclei; merge represents an overlay figure. (E) MTDH and CASC3 proteins were analyzed by western blot after overexpression of MTDH and CASC3 in MDA-MB-231 cells. (F) qPCR was used to measure circ-NOL10 binding to MTDH and CASC3 by using an antibody against FLAG pulldown bound complexes. Values were normalized to the level of background RIP, as detected by an IgG isotype control. (G) qPCR was used to measure circ-NOL10 binding to endogenous MTDH and CASC3 by using specificity antibody

[Legend continued on next page]
study found that expression of circ-NOL10 is significantly lower in breast cancer, especially in the LA and TNBC subtypes. Based on a large cohort of individuals with TNBC, we have shown that circ-NOL10 expression is correlated with clinical stage, lymphatic metastasis, recurrence, and survival. Downregulated circ-NOL10 is also associated with poor prognosis. Thus, circ-NOL10 can potentially be utilized as a promising diagnostic and prognostic biomarker for breast cancer.

Recently, a few RNA-binding proteins have been shown to control circRNA generation. For example, the splicing factor ESRP1 can interact with the flanking regions of circ-BIRC6-forming exons to promote formation of circ-BIRC6 in human embryonic stem cells.26 Another report found that ADAR1 mediates adenosine-to-inosine (A-to-I) RNA editing to inhibit circRNA production.22,27 However, the proteins that drive the circRNA differential expression in breast cancer remain elusive. We found that circ-NOL10 is highly inhibited in all subtypes of breast cancer compared with normal tissue. Previous reports have also indicated that circ-NOL10 is expressed at low levels in breast, lung, and colorectal cancer.28–30 This agreement suggests that circ-NOL10 is a common player in carcinogenesis. Investigation in lung cancer also found that circ-NOL10 is regulated by the splicing factor ESRP1. Although ESRP1 is indeed a candidate, based on our bioinformatics analysis of its expression profile and genomic variations, the experimental results did not support its role in regulation (Figure S3A). This reflected the flexible mechanistic option when cells facing different circumstance. Instead, our integrative approach identified two RBPs, MTDH and CASC3, that regulate circ-NOL10 expression in breast cancer.

MTDH was first identified as a mediator responsible for breast-to-lung cancer metastasis.31 Compared with non-malignant tissues, it is expressed highly in many cancers, including breast, prostate, and liver cancer.32–34 Subsequently, MTDH has been demonstrated to coordinate multiple signaling pathways implicated in various aspects of carcinogenesis and become an attractive novel therapeutic target.35–38 Limited biological function is known for CASC3 (also known as metastatic lymph node 51). It was first identified as core component of the exon junction complex.39 Previous studies have established that it resides in the chromosome 17q12–q21 region, where amplifications occur frequently.40 CASC3 overexpression has been found in breast cancer and fibroblast-like synoviocytes from individuals with rheumatoid arthritis.41,42 However, the role of CASC3 in breast cancer progression remains unclear. We found that these two RBPs bind and repress circ-NOL10, which is expressed at low levels in breast cancer. Thus, this investigation not only adds a novel ncRNA link to the many signaling pathways involving MTDH but also opens a new chapter for elucidating CASC3’s functional consequences.

This investigation of the regulation of circ-NOL10 and its biological functions and clinical implications in breast cancer should shed light on the circRNA-mediated mechanism in tumorigenesis.

**MATERIALS AND METHODS**

**Clinical samples**

16 normal mammary gland tissues and 178 breast cancer tissues were used in this study, which have been described previously in detail.13

**Cell culture**

MCF-7, MDA-MB-231, T47D, BT20, BT549, SKBR3, MDA-MB-157, MDA-MB-435, MDA-MB-436, MDA-MB-468, MCF-10A, and 293T cells were purchased from the ATCC and cultured under conditions recommended by the ATCC.

**Oligos, plasmids, and transfection**

siRNA oligonucleotides were designed and synthesized by Gene-Pharma (Suzhou, China). miRNA mimics or inhibitors were designed and synthesized by IGEbio (Guangzhou, China) (sequences are listed in Table S4). To efficiently circularize a circRNA transcript in cells, the circ-NOL10-overexpressing plasmid was synthesized by Genechem (Shanghai, China). The overexpression plasmids PEZ-93-MTDH-FLAG, pEZ-93-CASC3-FLAG, pEZ-93-SRSF1-FLAG, pEZ-93-ESRP1-FLAG, pEZ-93-CWC15-FLAG, pEZ-M14-ADRAR, and pEZ-M14-FUS were purchased from GeneCopoeia (Guangzhou, China). Cells were seeded in 6-well plates and cultured to 60%–70% confluence before transfection. Plasmids were transiently transfected using Lipofectamine 3000 (catalog number L3000015, Invitrogen, USA), and siRNA miRNA mimics, inhibitors, or corresponding controls were transiently transfected using Lipofectamine RNAiMAX (catalog number 13778150, Thermo Fisher Scientific, USA).

**RNA extraction and qPCR**

Total RNA was extracted using Trizol reagent (Life Technologies, Carlsbad, CA, USA). For circRNA and mRNA analyses, cDNA was synthesized using the PrimeScript RT reagent kit (Takara, Japan). Quantitative PCR was performed using SYBR Premix Ex TaqII (Takara, Japan), and the reactions were subsequently measured on an ABI7500 PCR instrument (Thermo Fisher Scientific, Waltham, MA, USA). GAPDH was applied as an internal standard control. For miRNA analyses, cDNA synthesis was performed with miRNA-specific stem-loop primers using a Quantscript RT Kit (Ibsbio, Guangzhou, China). SYBR Premix Ex TaqII (Takara, Japan) was used for transcript quantification with specific primers on a LightCycler 96 PCR instrument (Roche, Basel, Switzerland). Primers were designed according to our previous study and are listed in Table S4.13
**Protein isolation and western blot analyses**

Cells were lysed using RIPA lysis buffer (Biotecnology, China) containing a protease inhibitor cocktail (Roche, Switzerland). Proteins were separated by 10% SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA), incubated with 5% non-fat milk powder in TBST for 1 h at room temperature, and treated with specific primary antibodies overnight at 4°C. Then the membrane was incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody, and each band was detected using an enhanced chemiluminescence kit (Millipore, MA, USA) overnight at 4°C. The images were acquired on an Azure Imaging system (Azure, USA). Anti-CCBE1 was purchased from Biorbyt (catalog number orb215381). Anti-GAPDH, anti-caspase-3, and anti-cleaved caspase-3 antibodies were purchased from Cell Signaling Technology (catalog numbers 2118S, 9663S, and 9661S, respectively). TUSC2 antibodies were purchased from Affinity Bioscience (catalog number AF0500). Anti-glutamate receptor 3/GRIA3 [EP813Y] and anti-Bad were purchased from Abcam (catalog number ab40845 and ab40845, respectively). Anti-Glutamate receptor 3/3, and anti-cleaved caspase-3 antibodies were purchased from Cell Signaling Technology (catalog numbers 2118S, 9663S, and 9661S, respectively). TUSC2 antibodies were purchased from Affinity Bioscience (catalog number AF0500). Anti-glutamate receptor 3/GRIA3 [EP813Y] and anti-Bad were purchased from Abcam (catalog numbers ab40845 and ab40845, respectively). Anti-PDCD4 antibodies were purchased from Santa Cruz Biotechnology (catalog number sc-376430).

**RNA FISH**

FISH was performed as described previously in detail. Cy3-labeled probes specific to circ-NOL10 and fluorescein isothiocyanate (FITC)-labeled probes specific to 18S (Geneseed, Guangzhou, China; Table S4) were used in the hybridization. Nuclei were counterstained with 4,6-diamidino-2-phenylindole. The images were acquired on an Olympus FV3000 confocal microscope.

**RIP assays**

The RIP assays were performed using the Magna RIP RNA-Binding Protein Immuno-Precipitation Kit (Millipore, MA, USA). In brief, MDA-MB-231 cells were harvested when they reached 90%. 2 × 10^7 cells were washed in ice-cold PBS and resuspended in 100 μL of RIP lysis buffer combined with a protease inhibitor cocktail and RNase inhibitors. 100 μL cell lysate was incubated with beads coated with 5 μg of control mouse IgG or an antibody against CASC3 (Santa Cruz, Dallas, TX, USA) and an antibody against MTDH (Abcam, Cambridge, MA, USA) or against FLAG with rotation at 4°C overnight. After the lysates were treated with proteinase K buffer, immunoprecipitated RNA was extracted and reverse transcribed using the PrimeScript RT reagent kit (Takara, Japan). The abundance of circ-NOL10 was detected by qPCR. The immunoprecipitated RNAs were also sequenced on an Illumina NovaSeq 6000 system using the KAPA Stranded RNA-Seq Library Prep Kit (Illumina, USA). Motif analyses was conducted by TREME with default settings.43

**Dual-luciferase reporter gene assay**

The recombinant reporter plasmid (psiCHECK2_Firefly_Luciferase-Renilla_Luciferase containing the circ-NOL10 sequence psiCHECK2-NOL10), reporters containing circ-NOL10-luc with mutated MTDH binding sites (Luc-circ-N-mut A and Luc-circ-N-mut B), and circ-NOL10-luc with a mutated CASC3 binding site (Luc-circ-N-mut C) were designed by IGEbio (Guangzhou, China). For the miRNA-circRNA interaction assay, HEK293T cells were co-transfected with the reporter plasmid and miR-149-5p, miR-330-3p, miR-452-5p mimic, or a negative control mimic and incubated for 24 h. For the RBP-circRNA interaction assay, cells were co-transfected with the reporter plasmid and MTDH or CASC3 overexpression plasmids and incubated for 48 h. Then luciferase activity was detected with a dual-luciferase reporter assay kit (Beyotime Biotechnology, China).

**IF-FISH co-localization assay**

MDA-MB-231 cells were vaccinated in a confocal glass dish and fixed with 4% paraformaldehyde in PBS for 5 min. After washing three times with permeabilized with 0.1% Triton X-100 in PBS for 15 min, cells were added anhydrous ethanol 1 min and dried in the air. Cells were added with a denatured hybridization probe (Cy3-labeled probes specific to circ-NOL10, denatured at 88°C for 5 min, equilibrated at 4°C for 3 min) and incubated overnight at 37°C in a hybridization chamber. The next day, cells were rinsed with 2 × saline sodium citrate (SSC) preheated at 42°C for 5 min, and then rinsed with 2 × SSC at room temperature for 5 min twice. Then cells were blocked with 2% goat serum for 10 min at room temperature and incubated with primary antibody (MTDH antibody, Abcam, Cambridge, MA, USA and CASC3 antibody, Santa Cruz, Dallas, TX, USA) overnight at 4°C. On the third day, cells were washed with...
PBS and then incubated with the corresponding Alexa 488-labeled secondary antibody for 1 h at room temperature, followed by staining the nucleus with DAPI. Fluorescence images were acquired using an Olympus FV3000 confocal microscope. The colocalization overlap coefficient was analyzed using Olympus FV3000 software.

**Cellular assays**

**Wound healing assay**

MDA-MB-231 cells and MCF-7 cell were cultured in 6-well plates and transfected with a circ-NOL10-overexpressing plasmid or a control vector. The injury line was made with a 200-μL pipette tip when a monolayer of cells was plated in culture dishes at 100% confluence. Images of cell migration were captured at 0 and 24 h for MDA-MB-231 cells or at 48 h for MCF-7 cells. An average of eight random width of injury line was measured for quantization and normalized to the 0 h control and expressed as a relative migration rate. The assays were repeated at least three times.

**Colony formation assay**

Transfected MDA-MB-231 or MCF-7 cells were plated in 6-well plates at a density of 400 cells per well. Then cells were cultured for 14 days to form visible colonies. The cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet, and then colonies were imaged and counted. The experiment was replicated at least three times.

**Cell proliferation assay**

Each well of 2 × 10⁴ cells was seeded in five copies on a 96-well plate after MDA-MB-231 or MCF-7 cells were transfected for 48 h. 10 μL of CCK-8 reagent (Ycasen, China) was added to each well at 24, 48, 72, and 96 h and incubated at 37°C for 1 h. The optical density at 450 nm was measured using an automatic microplate reader (Synergy4, BioTek, Winooski, VT, USA). The experiment was repeated three times.

**Matrigel invasion assay**

Transfected cells were serum starved for 24 h, and 2 × 10⁴ MDA-MB-231 or MCF-7 cells in 200 μL serum-free medium were seeded in the upper chamber pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA). 600 μL of complete medium was added to the lower chambers, and then the cells were incubated for 24 h. Cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Invaded cells were counted in five random fields of view.

**Apoptosis assay**

Transfected cells were stained with PE7-AAD (catalog number 557963, BD Biosciences, CA, USA) and incubated 15 min after trypsinization. Then treated cells were analyzed by a FACSCANTO II flow cytometer and FlowJo software (BD Biosciences, San Jose, CA, USA).

**Lentiviruses and tumor xenograft model**

All animal experimental protocols were approved by the animal care and use Committee of Guangdong Medical University. A green fluorescent (ZsGreen) tagged circ-NOL10 OE vector (Lv-circ-NOL10) and blank vector (Lv-circ-control) were constructed and packaged in lentiviruses (HanBio, Shanghai, China). 4-week-old female nude mice were purchased from GemPharmatech (Nanjing, China) and kept under controlled conditions. Mice were divided randomly into 2 groups, with 5 mice in each group. Lv-circ-NOL10 and Lv-circ-control were transfected into MDA-MB-231 cells. Then MDA-MB-231 cells (2 × 10⁶ cells in 200 μL PBS solution) were inoculated subcutaneously into the left flanks of nude mice. The tumor volume was measured 1 week after injection and determined every 4 days, and the tumor volume (V = L × W²/2, L represents the longest diameter, and W represents the shortest diameter) was recorded each time. The mice were killed, and tumor tissue was removed for further research after 21 days.

**Bioinformatics analysis**

Potential miRNAs binding to circ-NOL10 are predicted by CircNet, CircInteractome, and Arraystar algorithms. We selected miRNAs that are expressed abundantly in breast cancer samples based on the miRGator database. To predict a potential regulator in circRNA biogenesis, 1,344 RBP reported in previous literature were used for this analysis. Multiple omics data were downloaded from the cbioPortal (http://www.cbioportal.org/), which includes somatic mutation, copy number variations, and differential mRNA expression in 825 breast-invasive carcinoma samples. The proportion of samples that harbors at least one of the above alterations was calculated. Furthermore, we ran a log rank test for each RBP to assess the association between gene alteration and survival time with R package “survival.” RBPs that are altered in more than 10% of all cancer samples and correlated with survival (p < 0.1) are reported as potential circRNA regulators in breast cancer.

**Statistical analyses**

Statistical analyses were performed using SPSS software with at least three independent experiments. The relationship between circ-NOL10 expression and clinical features was analyzed by independent t test. ROC curve analyses were performed using Prism 8.0 software (GraphPad, USA). DFS and OS curves were drawn using the Kaplan-Meier method. Univariate and multivariate Cox regression models were used to evaluate risk factors for breast cancer DFS and OS.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.09.013.

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**AUTHOR CONTRIBUTIONS**

J.X. and M.C. conceived the project and designed the experiments. Y.C., X.Z., Q.C., C.-C.S., Y.-X.O., J.F., and L.C. performed the
experiments. X.Z. and F.Z. contributed to acquisition and analysis of clinical data. J.X. and D.C. developed the computational pipeline. J.X. and M.C. collected and interpreted the data. J.X. wrote the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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