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FOXO1 3′UTR functions as a ceRNA in repressing the metastases of breast cancer cells via regulating miRNA activity

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ABSTRACT

The competitive endogenous RNAs (ceRNAs) are RNA molecules that affect each other's expression through competition for their shared microRNAs (miRNAs). In this study we explored whether FOXO1 3′UTR can function as a ceRNA in repressing epithelial-to-mesenchymal transition (EMT) and metastasis of breast cancer cells via regulating miR-9 activity. We found that miR-9 binds to both the FOXO1- and E-cadherin-3′UTR, indicating that the FOXO1- and E-cadherin-3′UTR can be linked through miR-9. Follow-up analyses showed that there existed a competition of miR-9 between FOXO1 and E-cadherin-3′UTR. Thus FOXO1 3′UTR inhibits the metastases of breast cancer cells via induction of E-cadherin expression. Our results suggest that FOXO1 3′UTR may function as a miRNA-inhibitor in modulating metastasis of breast cancer cells.
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1. Introduction

Breast cancer, one of the most common malignancies, is the second leading cause of cancer death in women [1]. According to previous researches, the metastasis of cancer cells is responsible for majority of the deaths of breast cancer patients, and therefore is regarded as the primary clinical challenge of breast cancer [2]. Two hypotheses have been postulated to explain the intricate nature of the metastatic process: (1) tumor stem cells, a population of cells within the tumor mass, are able to proliferate, self-renew and induce tumorigenesis, (2) epithelial-to-mesenchymal transition (EMT), in which the epithelial cancer cells switch from a well-differentiated phenotype to an invasive mesenchymal state in pathological conditions [3].

MicroRNAs (miRNAs) are negative regulators of gene expression by decreasing the stability of target RNAs or limiting their translation [4]. Most evidence indicated that miRNAs could be both positive and negative regulators of cancer metastasis by regulating the process of EMT [5]. And recent study showed that miR-9 could promote the progression of metastasis in breast cancer through regulating E-cadherin expression directly and promoting EMT [6]. Previous studies showed that the expression of 3′UTRs could act as competing endogenous RNAs (ceRNAs) in regulating the function of endogenous miRNAs [7–11]. Conversely, protein-coding transcripts, especially transcription factors like FOXO1, can bind and regulate the function of miRNA [12], thus, these transcripts regulate each other in the ceRNA networks [13–15].

FOXO1 belongs to the FOXO family of Forkhead transcription factors, which have a conserved DNA binding domain termed the Forkhead box (Fox) [16]. Increasing evidence suggests that FOXO1 is also involved in tumor metastases [17,18]. And restoring or targeting FOXO1 to the cell nucleus in specific tissues may improve the prognosis of breast cancer [19]. Some studies showed that FOXO1 3′UTR can be regulated by endogenous miRNAs, and the regulation is coordinated by a group of miRNAs (miR-27a, miR-96, and miR-182) in breast cancer cells [20]. However, it is unclear whether FOXO1 3′UTR can function as a ceRNA in regulating mature miRNAs in breast cancer cells.

In this study, we confirmed that FOXO1 3′UTR was a direct target of miR-9. Wound healing, Transwell Migration assay and Adhesion Assay showed the suppression function of FOXO1 3′UTR on the metastases of breast cancer cells. Furthermore, we proved the existence of a competition of miRNAs between FOXO1 3′UTR and E-cadherin 3′UTR. To our knowledge, this is the first study demonstrating that the FOXO1 3′UTR acts as E-cadherin ceRNA

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and inhibits metastasis by arresting miR-9 function in breast cancer cells. Therefore, this study will provide novel perspectives into the diagnosis and treatment of breast cancer.

2. Materials and methods

2.1. Cell culture

Human breast cancer cells MCF-7, MDA-MB-231, MDA-MB-453, SKBR3 and normal breast epithelial cells MCF-10A were obtained from the ATCC (Manassas, Virginia, USA). MCF-7, MDA-MB-231, MDA-MB-453 and MCF-10A cells were cultured in the Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY) at 37 °C in a humidified atmosphere with 5% CO_2. SKBR3 was cultured in RPMI 1640 (Gibco, Grand Island, NY) media with 10% FBS at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Plasmid construction

FOXO1 3′UTR and E-cadherin 3′UTR (CDH1 3′UTR), which has miR-9 binding sites were amplified by polymerase chain reaction (PCR) from human cDNA using the following primers:

FOXO13′UTR: forward, CGCGGATCCGGGTTAGTGAGCAGGTTACACTT;
reverse, CCCAAGCTTTTCATTGTAATGAAATTTCCAATGG,
CDH13′UTR: forward, CGCGGATCCCTGATGCAGAAATTATTGGGCTC;
reverse, CCCAAGCTTTGTTCAGCTCAGCCAGCATTT.

Then the PCR products were cloned into the pSilencer-4.1 vector (Ambion, Austin, USA), and the constructs were verified by DNA sequencing. These recombinant plasmids were denoted FOXO1 3′UTR and CDH1 3′UTR.

2.3. Transfection

Cells at the density of 5×10^5/well were harvested and seeded in a 6-well plate. Four micrograms of DNA or 50 nM of RNA (miR-9 mimics; miR-9 inhibitor; miRNA NC; FOXO1 siRNA; Dicer siRNA; siRNA NC) (Biomics Biotechnology Inc., China) were transfected into the cells by using the Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA), following the manufacturer's protocol.

2.4. Total RNA extraction and quantitative real time PCR (qRT-PCR)

Total RNA was extracted using the Trizol reagent (Invitrogen, USA). Synthesis of first strand cDNA was performed with M-MLV (Promega, USA) following standard protocols. EzOmics SYBR qPCR, miRNA qRT-PCR kit and miR-9 primer, which were purchased from Biomics, were performed in a qRT-PCR detection system (ABI, USA). The miR-9 expression level was normalized to U6 snRNA, then the miR-9, FOXO1 and CDH1 relative expression level of each group were calculated using the $2^{-\Delta\Delta ct}$ method.

2.5. Wound healing assays

MCF-7 and MDA-MB-231 cells were transiently transfected with FOXO1 3′UTR, CDH1 3′UTR and FOXO1 siRNA in a 6-well plate. After 24 h, cells were allowed to grow to 90% – confluent in complete medium. Cell monolayers were wounded with a sterile micropipette tip, and wounded monolayers were washed with phosphate buffer solution (PBS) for several times to remove cell debris, and then serum-free medium was added for further incubating. The images of scratch were captured, and the distances between two edges were scaled for three positions each time at over three time points. Since MDA-MB-231 cells migrate faster than MCF-7 cells, MDA-MB-231 and MCF-7 cells were measured every 12 h and every 24 h respectively.

2.6. Transwell migration assay

Transwell migration assays were carried out by using 24-well MILLIcell Hanging Cell Culture inserts 8 mm PET (MILLIPORE), which were coated with Matrigel matrix gel (BD Biosciences). After transfection, a total of 10^5 cells in serum-free medium were added to the upper chamber and allowed to migrate toward the bottom chamber. Twenty percentage FBS was used as a chemo-attractant in the medium of the bottom chamber. MDA-MB-231 cells were stopped at 10 h, and MCF-7 cells were stopped at 20 h. Cells on the top surface of the membranes were removed with a cotton swab, and cells on the underside were fixed in methanol and stained with 0.1% viola crystalline solution. Five random fields from each of the triplicate migration assays were counted by using phase contrast microscopy. Quantification was done by measuring with Microplate Reader (OD 570 nm) after being destained with glacial acetic acid.

2.7. Adhesion assay

Microtiter wells were coated with matrix gel (BD Biosciences) 37 °C, 4 h. Then the wells were blocked for 1 h with 1% BSA in PBS. Transfected cells were trypsinized and suspended at a final concentration of 10^5 cells/well in serum-free medium. After 1 h adhesion, non-adherent cells were washed away with PBS. The colorimetric MTT-assay was used to determine the number of adherent cells.

2.8. Luciferase reporter assay

pMiR-Report Fluc vectors (Ambion) were used to introduce the portion of the 3′UTR of FOXO1 and CDH1 mRNA, the wild-type and mutant binding sites for miR-9. These fragments were synthesized by Sangon Biotech (Shanghai, China) and annealed as described. The annealed oligonucleotides were ligated into pMiR-Report. In order to test whether miR-9 were bounded directly to the 3′UTR of FOXO1 and E-cadherin, MCF-7 and MDA-MB-231 cells were seeded in a 24-well plate (4×10^4 cells/well) and co-transfected with pMiR-Report wild-type (WT) or mutant (MUT) sequences (denoted as FW; FM; CW; CM), miR-9 mimics or cel-67 mimics and pMiR-Report β-gal control plasmid (Ambion) using Lipofectamine 2000. After 48 h transfection, cells were collected and luciferase activity was measured by a Glomax 96 luminometer (Promega). The transfection efficiency was normalized to β-galactosidase activity. In order to study whether FOXO1 3′UTR or E-cadherin 3′UTR could competingly bind endogenous miR-9, MCF-7 and MDA-MB-231 cells were seeded in 24-well tissue culture plates and co-transfected with the luciferase reporter constructs (FW; FM; CW; CM) and the 3′UTR constructs (FOXO1 3′UTR and E-cadherin 3′UTR) by using Lipofectamine 2000. Cells were collected and measured by applying the method described after transfection.

2.9. Western blot

After treatment, cells were washed twice with ice-cold PBS and lysed in buffer RIPA (Beyotime, China), which contains 1 mM PMSF (Beyotime), for 30 min (vortexed every 5 min) on ice, and then centrifuged (400×g) at 4 °C for 10 min. The supernatant was separated by 10% SDS–PAGE and transfered onto polyvinylidinedifluoride membranes. The membranes were blocked in 5% BSA and

incubated with appropriate primary antibodies overnight at 4 °C washed and then incubated with horseradish peroxidase labeled secondary antibodies for 2 h at room temperature. The FOXO1, E-cadherin, vimentin, α-SMA, N-cadherin and Dicer antibody were purchased from Epitomics and β-actin was purchased from Bio-world. The specific protein bands were detected using the ECL chemiluminescence detection kit (Beyotime, China), followed by exposure to Kodak X-ray films subjected to SDS–PAGE. The protein expression level was normalized to β-actin.

2.10. Immunofluorescent assay

Cells were grown on glass chamber slides, fixed with 4% para-formaldehyde in PBS for 30 min, permeabilized in 0.1% Triton X-100 for 30 min and blocked with 3% BSA in PBS for 1 h at room temperature. The cells were incubated with rabbit anti-human E-cadherin and anti-vimentin antibody (Epitomics, Burlingame, CA) overnight at 4 °C. Then, the cells were incubated with FITC-conjugated secondary antibody (Bioworld, Atlanta, Georgia, USA) for 1 h and observed with the Laser scanning confocal microscope. The cells were washed with PBS after incubating each time.

2.11. Statistical analysis

The experimental results were shown as the mean ± S.E.M. for three independent experiments. The differences between the groups were analyzed using a two-sided Student's *t*-test, and $P < 0.05$ was considered significant.

3. Results

3.1. FOXO1 was coexpressed with E-cadherin and both were regulated by common miRNA in MCF-7 and MDA-MB-231 cells

We detected the expression of FOXO1 and E-cadherin in MCF-7, MDA-MB-453, SKBR3, MDA-MB-231 and MCF-10A cells in mRNA and protein levesl by using qRT-PCR and Western blot respectively. Results in Fig. 1A showed that the expression of FOXO1 in MCF-7, a less metaststic tumor cell line, was 3.33-fold more than MDA-MB-231, a highly metastatic tumor cell line. The expression level of E-cadherin was similar to the expression level of FOXO1 in MCF-7 and MDA-MB-231 cells (Fig. 1A–C). We also looked at the expression of miR-9 in MCF-7, MDA-MB-453, SKBR3, MDA-MB-231 and MCF-10A cells. The expression of miR-9 in MDA-MB-231 was 5.34-fold more than in MCF-7 cells (Fig. 1D). We found out that miR-9 has putative target sites on both FOXO1 and E-cadherin by using TargetScan 6.1 (www.targetscan.org) (Fig. 1E). To validate whether FOXO1 and E-cadherin are bona fide targets of miR-9, human FOXO1 and E-cadherin 3′UTR fragments containing wild-type or mutant miR-9 binding site were introduced downstream of the luciferase reporter gene. When miR-9 mimics or cel-67 mimics were cotransfected with the reporter plasmid, the relative luciferase activity of the reporter containing wild-type FOXO1 and E-cadherin 3′-UTR were markedly suppressed, while the luciferase activity of the reporter containing mutant 3′-UTR was unaffected. It indicated that FOXO1 and E-cadherin were direct targets of miR-9 (Fig. 1F and G). Results in Fig. 1H and I showed that the

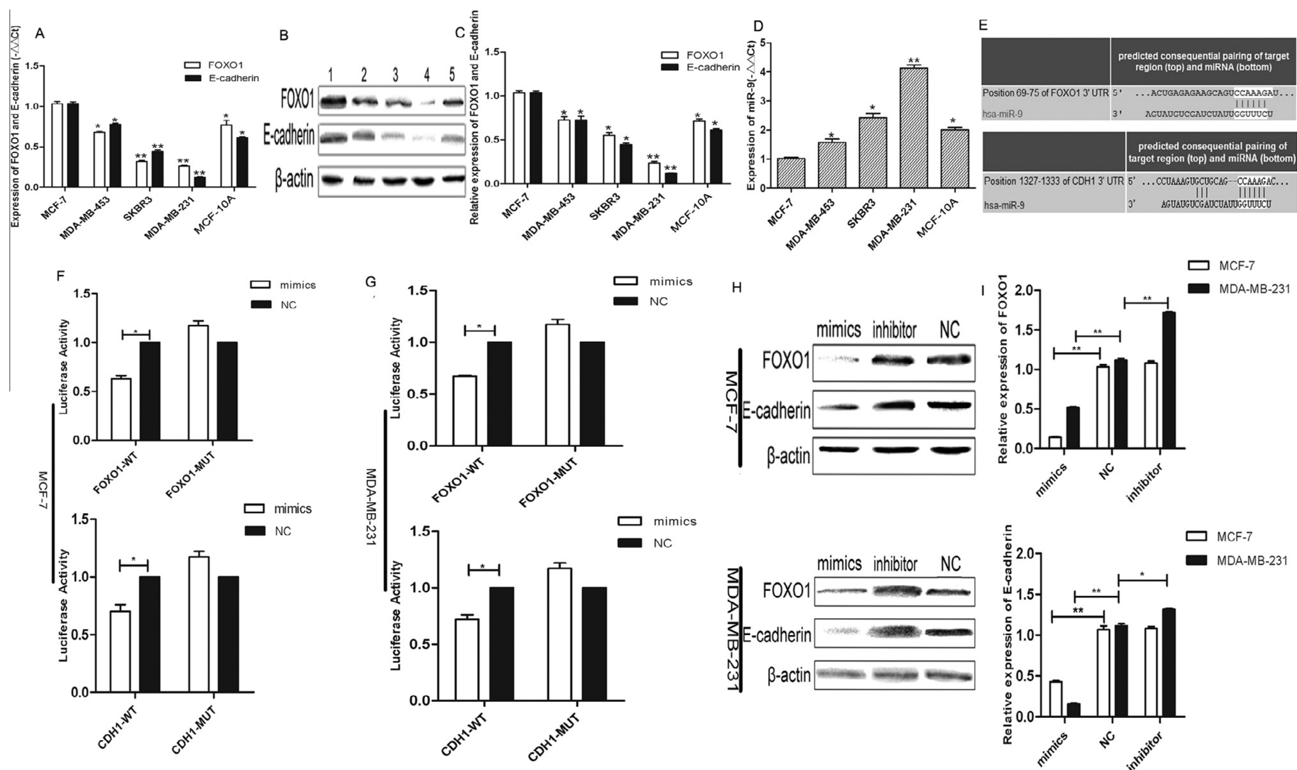

Fig. 1. FOXO1 was coexpressed with E-cadherin and both were regulated by common miRNA in MCF-7 and MDA-MB-231 cells. (A–C) Expression of FOXO1 and E-cadherin in MCF-7, MDA-MB-453, SKBR3, MDA-MB-231 and MCF-10A cells measured by qRT-PCR and Western blot. (Fig. 1B, 1. MCF-7, 2. MDA-MB-453, 3. SKBR3, 4. MDA-MB-231, 5. MCF-10A) The normalized FOXO1 and E-cadherin expression for MCF-7 was set 1, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. MCF-7. (D) Expression of miR-9 in MCF-7, MDA-MB-453, SKBR3, MDA-MB-231 and MCF-10A cells measured by real-time RT-PCR. The normalized miR-9 expression for MCF-7 was set 1. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. MCF-7. (E) The miR-9 targets on FOXO1 and E-cadherin were predicted using Targetscan 6.1. (F, G) Luciferase reporter assay after transfection. Fluc activity in cells was measured and normalized to β-galactosidase activity. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. NC group. (H) FOXO1 and E-cadherin protein after transfecting with miR-9 mimics; inhibitor and NC were detected by Western blot. (I) Quantitative data of densitometric analyses. The relative expression of FOXO1 and E-cadherin was normalized to β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. NC group.

expression of FOXO1 and E-cadherin were reduced by almost half in MCF-7 and MDA-MB-231 cells after transfection with miR-9 mimics, whereas, miR-9 inhibitor increased the expression of FOXO1 and E-cadherin in MDA-MB-231 cells and had little effect on FOXO1 and E-cadherin expression in MCF-7 cells. This may be due to a low level of endogenous miR-9 in MCF-7 cells. It revealed that miR-9 could modulate FOXO1 and E-cadherin expression in a post-transcriptional manner.

3.2. FOXO1 3′UTR suppressed metastasis of MCF-7 and MDA-MB-231 cells

In order to examine the effect of FOXO1 3′UTR on the metastasis of MCF-7 and MDA-MB-231 cells, we overexpressed and knocked down FOXO1 3′UTR in MCF-7 and MDA-MB-231 cells. The efficiency of transfection was confirmed by qRT-PCR and Western blot (Fig. 2F and L). The motility of MCF-7 and MDA-MB-231cells was examined by wound healing assay. MCF-7 and MDA-MB-231 cells were transfected with FOXO1 3′UTR and vertical migration was assessed. A significant difference in cell migration was observed between FOXO1 3′UTR and control vector transfected breast cancer cells. Cell migration was decreased in FOXO1 3′UTR-transfected cells compared with controls (Fig. 2A and B). To find out whether there is a correlation existed between FOXO1 3′UTR and tumor invasiveness, MCF-7 and MDA-MB-231 cells were transfected with either FOXO1 3′UTR or control vectors. As shown in Fig. 2C, we found that invasive activity of FOXO1 3′UTR-transfected MCF-7 and MDA-MB-231 cells was markedly attenuated compared to vector control (Fig. 2C and D). We also examined the influence of FOXO1 3′UTR on the adhesion activities of breast cancer MCF-7 and MDA-MB-231 cells to the substrates precoated with matrix gel, which is a basement member component. After transfected with FOXO1 3′UTR, the adhesion activity was decreased in MCF-7 and MDA-MB-231 cells (Fig. 2E). Following FOXO1 3′UTR knock down, the cell migration adhesion and invasion phenotypes were significantly increased compared to the treatment of negative control (NC) in MCF-7 and MDA-MB-231 cells (Fig. 2G–K). In summary, these results showed that FOXO1 3′UTR expression can inhibit metastasis of MCF-7 and MDA-MB-231 cells.

3.3. FOXO1 3′UTR promoted E-cadherin expression, thus inhibiting EMT in MCF-7 and MDA-MB-231 cells

Our experiments showed that the FOXO1 3′UTR transfected cells expressed higher levels of E-cadherin compared to vector control (Fig. 3A). E-cadherin expression in MCF-7 and MDA-MB-231 cells was increased by 2.30 and 2.22-fold separately (Fig. 3B). However, The expression of E-cadherin in FOXO1 siRNA transfected MCF-7 and MDA-MB-231 cells was reduced by 2.52 and 2.43-fold separately compared to NC (Fig. 3C and D). The currently and commonly used biomarkers of mesenchymal cells such as N-cadherin, vimentin and a-SMA were used to identify the EMT phenomenon. Protein levels of N-cadherin; vimentin and a-SMA were reduced in FOXO1 3′UTR transfeted cells (Fig. 3E and F). In contrast, knock

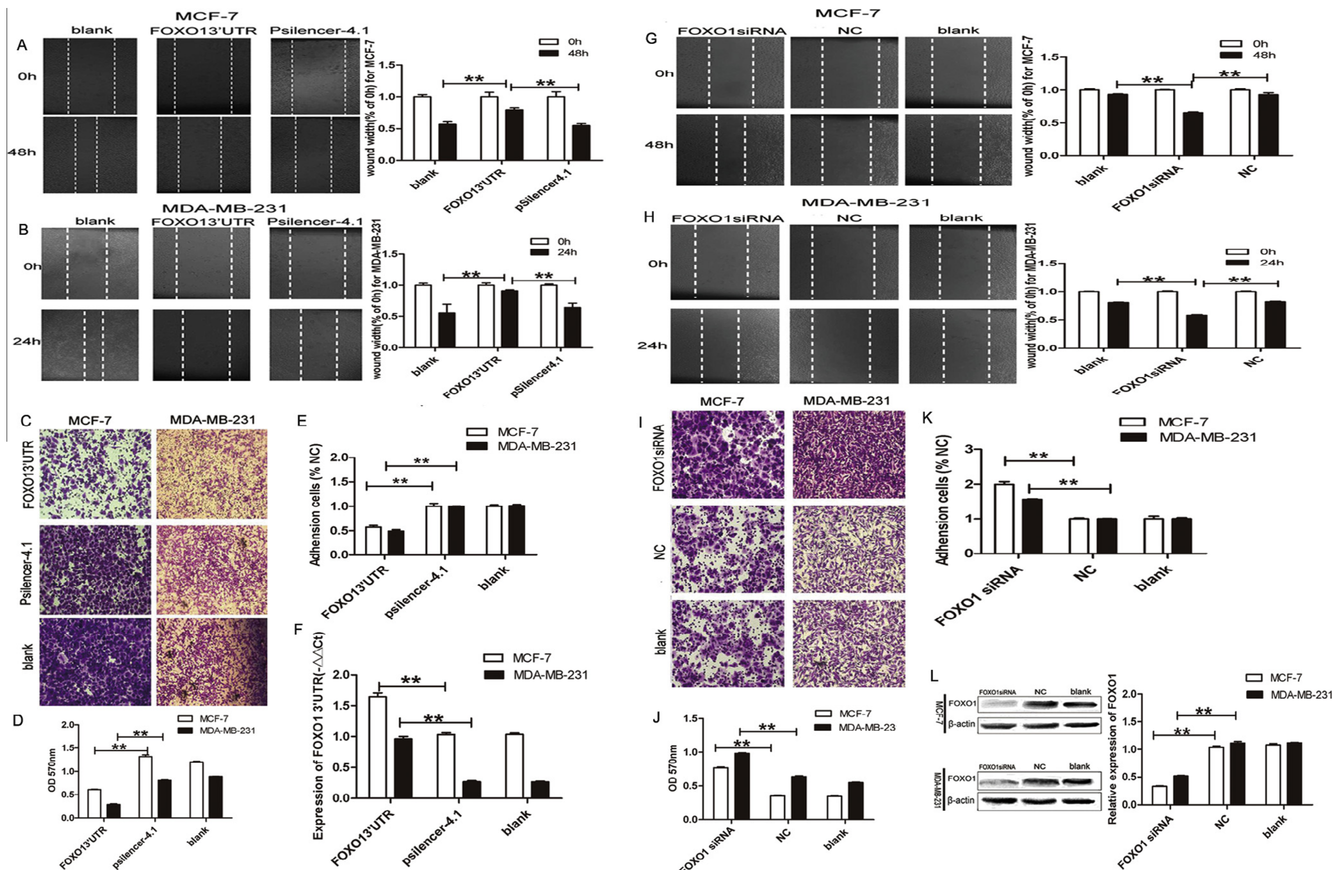

Fig. 2. FOXO1 3′UTR suppressed metastasis of MCF-7 and MDA-MB-231 cells. (F, L) The efficiency of transfection was confirmed by qRT-PCR and Western blot. $^{**}P < 0.01$ vs. NC group. (A, B) Effects of FOXO1 3′UTR on MCF-7 and MDA-MB-231 cells migration in vitro. $^{**}P < 0.01$ vs. Control group. (C, D) Effects of FOXO1 3′UTR on cell invasion in MCF-7 and MDA-MB-231 cells (C) Photographs of the cell invasion through the polycarbonate membrane stain by crystal violet (200×). (D) OD570 of stain crystal violet. (E) Effects of FOXO1 3′UTR on adhesion to matrix gel of MCF-7 and MDA-MB-231 cells. $^{**}P < 0.01$ vs. NC group. (G, H) Effects of FOXO1 siRNA on cell migration in MCF-7 and MDA-MB-231 cells. (I, J) Effects of FOXO1 siRNA on cell invasion in MCF-7 and MDA-MB-231 cells. (I) Photographs of the cell invasion through the polycarbonate membrane stained with crystal violet (200×). (J) OD570 of stain crystal violet. (K) Effects of FOXO1 siRNA on adhesion to matrix gel of MCF-7 and MDA-MB-231 cells. $^{**}P < 0.01$ vs NC group.

down of FOXO1 3′UTR in MCF-7 and MDA-MB-231 cells caused a significant increase in the expression of mesenchymal markers (Fig. 3G and H). Meanwhile, immunofluorescence assay showed that the expression of E-cadherin was up-regulated in MCF-7 and MDA-MB-231 cells when treated with FOXO1 3′UTR (Fig. 3I). Whereas, vimentin expression was down-regulated in FOXO1 3′UTR transfected breast cancer cells compared with control (Fig. 3J). The morphology of cells treated with FOXO1 3′UTR changed from long shuttleshape to spherical, especially in MDA-MB-231 cells, which have higher metastasis potential.

3.4. Regulation of E-cadherin by FOXO1 3′UTR was miRNA dependent in MCF-7 and MDA-MB-231 cells

To decipher the nature of the relationship between FOXO1 3′UTR and E-cadherin 3′UTR, we expressed FOXO1 3′UTR luciferase reporter (FW) or E-cadherin 3′UTR luciferase reporter (CW) in MCF-7 and MDA-MB-231 cells, the results showed that overexpression of FOXO1 3′UTR significantly increased E-cadherin 3′UTR luciferase reporter (CW) activity, but the activity of FOXO1 3′UTR luciferase reporter (FW) remained unaltered in E-cadherin 3′UTR overexpressing cells (Fig. 4A). However, FOXO1 3′UTR mediated increase of E-cadherin activity was lost when Dicer was knocked down. E-cadherin expression was not altered when we cotransfected FOXO1 3′UTR with Dicer siRNA (Fig. 4B and D). When FOXO1 siRNA was co-expressed with Dicer siRNA, the expression of E-cadherin stayed the same in MCF-7 and MDA-MB-231 cells (Fig. 4C and E). The efficiency of transfection was confirmed by western blot (Fig. 4F and G).

4. Discussion

In this study, we provided evidence that FOXO1 3′UTR can function as a ceRNA in competitively binding with miR-9, and therefore relieve the suppression of E-cadherin from miR-9 and up-regulate the expression of E-cadherin in breast cancer cells. E-cadherin is a cell–cell adhesion molecule that forms complexes with catenin family members and regulates association with the cytoskeleton and catenin/WNT signaling pathways. It is a critical molecule in the EMT process. The metastases are correlated with the low level of E-cadherin occurring in the EMT [21]. A study suggested that the disruption of E-cadherin junctions and consequent gain of cell motility contribute to the process known as EMT in breast cancer cells. Conversely, overexpression of E-cadherin suppresses tumor invasion [22–24]. Thus, our results suggested that FOXO1 3′UTR suppressed the metastases of MCF-7 and MDA-MB-231 cells by promoting the expression of E-cadherin and repressing EMT.

The prevalent view is that protein-coding gene must be translated into a protein to exert function. FOXO1-coded protein, which acts as a key tumor suppressor, has been extensively studied [25–27]. Previous studies showed that FOXO1 participates in many biological events [28–32]. Recent studies indicated that miRNAs modulate FOXO1 expression through binding to FOXO1 3′UTR, which has miRNA response elements (MRES) in breast cancer cells [20]. However, there have been few reports about the regulation of FOXO1 3′UTR on miRNAs. Here we used ceRNA hypothsis to explain the regulatory function of FOXO1 3′UTR. Competing endogenous RNA (ceRNA) hypothesis, which had been proved in recent studies, is a unifying hypothesis about how messenger RNAs,

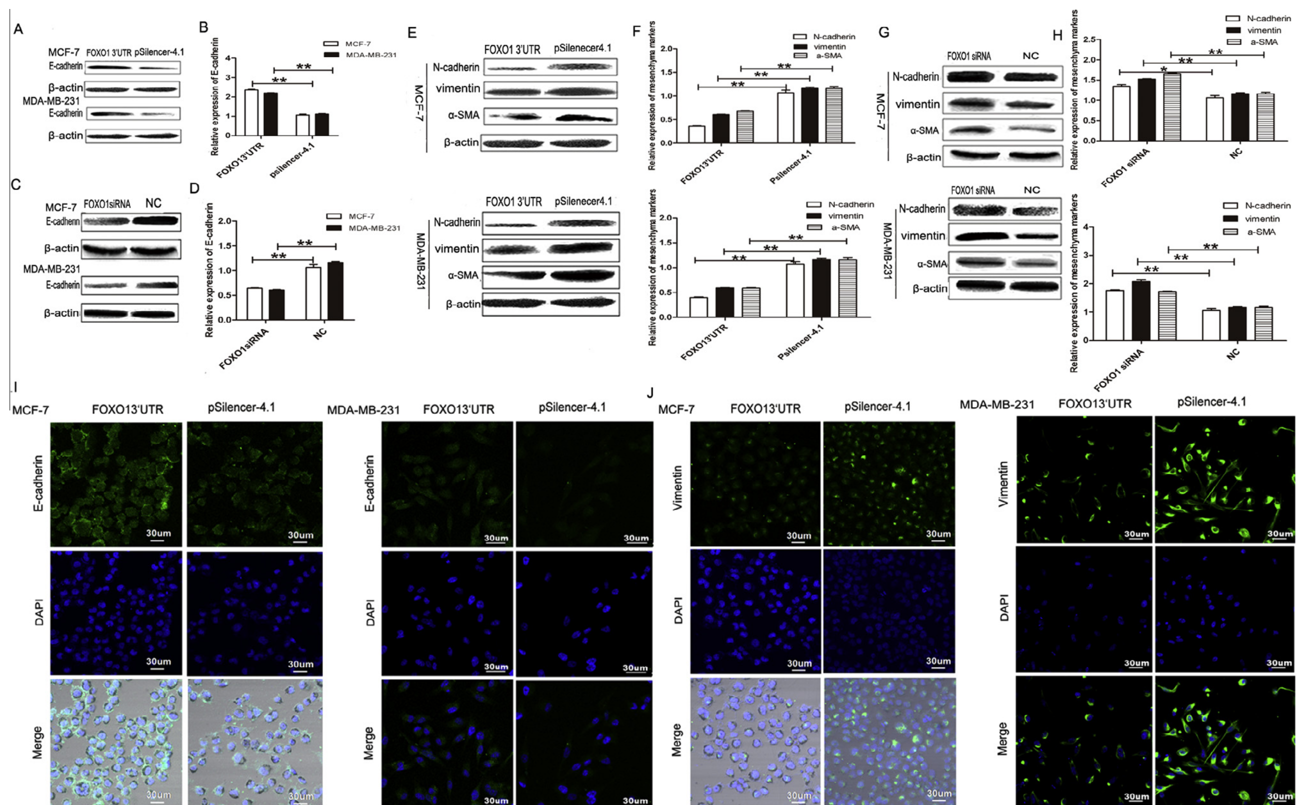

Fig. 3. FOXO1 3′UTR promoted E-cadherin expression, thus inhibiting EMT in MCF-7 and MDA-MB-231 cells. (A, B) Western blot analysis of MCF-7 and MDA-MB-231 for E-cadherin protein expression after transfection with FOXO1 3′UTR, The relative expression of E-cadherin was normalized to β-actin. Increased E-cadherin expression was detected in FOXO1 3′UTR transfected cells. $^{**}P < 0.01$ vs. NC group. (C, D) After FOXO1 3′UTR being koncked down, E-cadherin expression was detected by western blot. The relative expression of E-cadherin was normalized to β-actin. $^{**}P < 0.01$ vs. NC group. (E–H) FOXO1 3′UTR affected vimentin; N-cadherin and α-SMA at protein level. (E, G) Expression of vimentin; N-cadherin and α-SMA by western blot with overexpression or knockdown of FOXO1 3′UTR. (F, H) Representative quantitative data of densitometric analyses. $^{**}P < 0.01$ vs. NC group. (I, J) Immunofluorescence analysis of E-cadherin and vimentin expression after transfecting FOXO1 3′UTR. Green, E-cadherin and vimentin were immunostained with anti-E-cadherin and antin-vimentin, blue, nuclei were stained with DAPI.

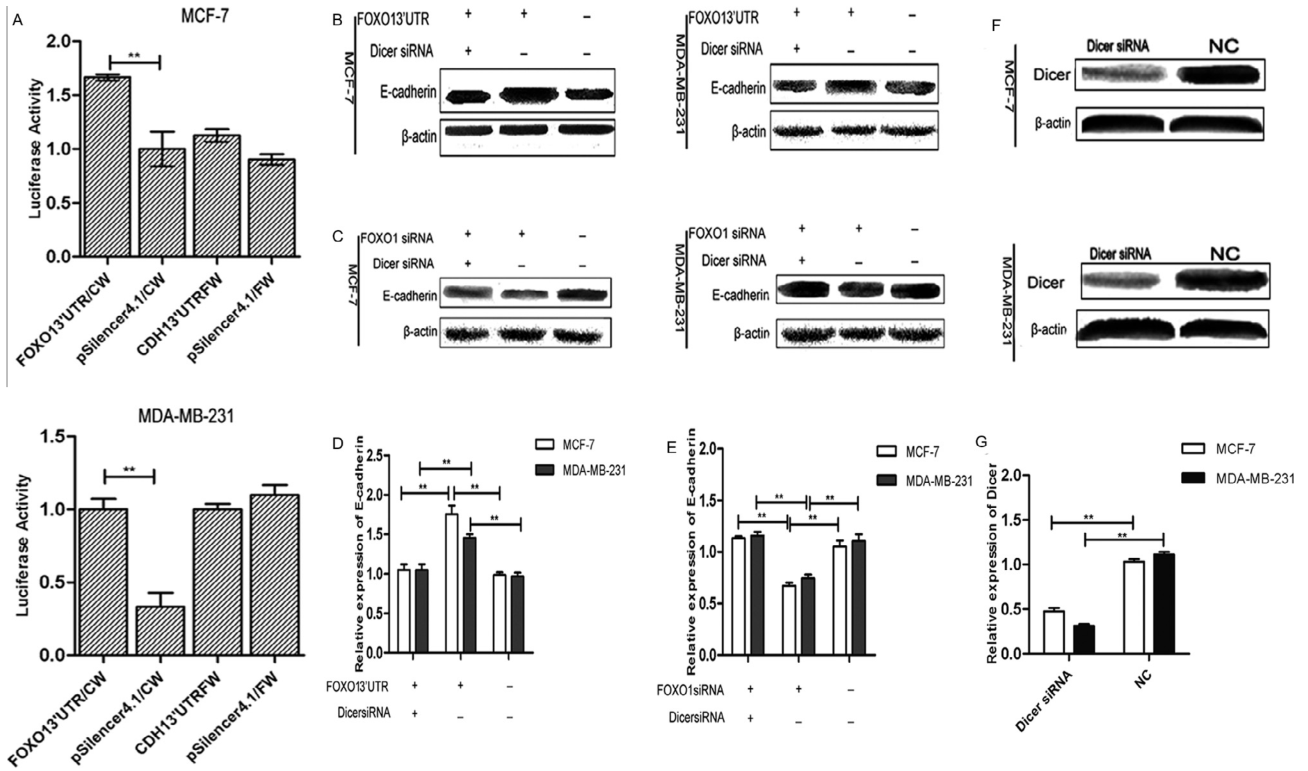

Fig. 4. Regulation of E-cadherin by FOXO1 3′UTR is miRNA dependent in MCF-7 and MDA-MB-231 cells. (A) FOXO1 3′UTR and E-cadherin 3′UTR luciferase activity in MCF-7 and MDA-MB-231cells transfected with the 3′UTR of negative control (pSilencer 4.1), FOXO1 or E-cadherin 3′UTR. $^{**}P < 0.01$ vs. NC group. (B) When added FOXO1 3′UTR plus Dicer siRNA, E-cadherin expression was not altered. (C) When FOXO1 siRNA was co-expressed with Dicer siRNA, the expression of E-cadherin stayed the same in MCF-7 and MDA-MB-231 cells. (D, E) Representative quantitative data of densitometric analyses. $^{**}P < 0.01$ vs. NC group. (F, G) The efficiency of transfection was confirmed by Western blot. $^{**}P < 0.01$ vs. NC group.

transcribed pseudogenes, and long non-coding RNAs (lncRNAs) regulate each other's expression by miRNA response elements (MREs) to compete for the binding of miRNAs [21–25]. Throuth a series of experiments, our research confirmed that FOXO1 3′UTR can function as a ceRNA of E-cadherin and act in trans to modulate E-cadherin levels by competitively binding to miR-9 in breast cancer cells. However, other study showed that FOXO1 directly regulated matrix metalloprotease 1 transcription, and Cdc25A influenced matrix metalloprotease 1 through regulating nuclear localization and stability of FOXO1 [33]. This study found that FOXO1 has a role of pro-metastasis in breast cancer cells. These controversial findings revealed that non-coding function of mRNA might be adverse to the coding function, thus creating functional complexity and diversification in both physiological and pathological conditions. Accordingly it is important to study non-coding function of mRNA.

Our results proved that expressing non-coding transcript may have a dramatic function of regulating gene expression. Exogenous expression of the 3′UTR construct altered the expression of some proteins that are functionally associated with the 3′UTR. For our study, we constructed FOXO1 3′UTR that only contained miR-9 binding site. However, computational analysis of the FOXO1 3′UTR has shown that miR-27a, miR-96 and miR-182 could interact with FOXO1 3′UTR. Recent studies have reported that miR-27a, miR-96 and miR-182 have oncogenic activities in breast cancer [34–36]. Long sequence of the 3′UTR that contains miR-27a, miR-96 and miR-182 binding sites can be constructed for further research. Its capacity of exerting complex biological activities may be observed. We predict that the effectiveness of 3′UTR will depend on the number of miRNAs that it can "sponge" which arrest miRNA activity. Further experiments are needed to prove our hypothesis. Although a ceRNA networks can be built around a single miRNA, more attention should be paid on the most robust ceRNA network that contains transcripts, which share multiple MREs targeted by multiple miRNAs. CeRNA networks may depend on the identity, concentration, and subcellular distribution of the miRNA species.

Our results showed that the expression of the 3′UTR could produce a similar functional role as the miRNA inhibitor. However, 3′UTR has a more potent effect compared to miRNA inbibitor in some aspects. Firstly, one 3′UTR has the capacity to modulate multiple miRNAs, while one miRNA inhibitor can only affect one miRNA. Secondly, a long fragment of 3′UTR seems more stable than the miRNA inhibitor, which may be readily degraded. Therefore, the expression of a 3′UTR may have a greater advantage in modulating cell activities compared to miR-9 inhibitor, providing a potential use for 3′UTR transcript in gene therapy.

Our results showed that FOXO1 3′UTR overexpression significantly increased the activity of E-cadherin 3′UTR luciferase reporter, while the activity of FOXO1 3′UTR luciferase reporter was not dramatically altered in E-cadherin 3′UTR overexpressing cells. This result suggested the superiority of FOXO1 3′UTR in the competition of miRNA, in comparison to E-cadherin 3′UTR. Our results indicated that not all of the MREs on ceRNAs are equivalent. The ceRNA effect is known to be determined by not only the sequence dependent binding affinity, but also the relative concentration of the ceRNAs. Therefore, the MRE's nucleotide composition of ceRNAs is critical for the overall ceRNA function when its concentration stays unchanged. Some studies tried to assess the probability of interaction between two moleculars via mathematical/computational analysis [37]. However, no further evidence has been given to support these theories. Therefore, it is necessary to develop more practical methods to solve these questions. Interestingly, Our results

showed that E-cadherin expression was higher in FOXO1 3′UTR overexpressing MCF-7 cells than that in MDA-MB-231 cells. Here, FOXO1 3′UTR acted as a ceRNA of E-cadherin. The effectiveness of a ceRNA depends on not only its expression, but also the ceRNA's accessibility to miRNA molecules, which is influenced by its subcellular localization and its interaction with RNA-binding proteins. Thus, we suppose that the affinity between FOXO1 3′UTR and miR-9 in MCF-7 cells might be stronger than that in MDA-MB-231 cells, because different subtypes of cells might have various internal microenvironment. We suggest that this stronger affinity might explain that E-cadherin expression was higher in FOXO1 3′UTR overexpressing MCF-7 cells. However, this hypothesis needs further verification. CeRNA networks are very complicated gene regulatory networks and might be influenced by multiple factors. Only when we solve all these problems and do more complete study can we fully utilize the ceRNA networks to defeat disease.

All together, our study suggested that FOXO1 3′UTR may be a promising miRNA-inhibitor, which can be applied in the gene therapy for breast cancer. Moreover, ceRNA networks might provide new perspectives into the diagnosis and treatment of disease. Although the exploration and studies in this field are still in their infancy, we believe that ceRNA network will be thoroughly understood and present opportunities for new therapies with the progress of experimental tools.

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