

Original Article

FOXO1 and hsa-microRNA-204-5p affect the biologic behavior of MDA-MB-231 breast cancer cells

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Abstract: RNA molecules and targeting microRNA (miRNA) have been reported as novel focuses in recent research on breast cancer. This study aimed to probe the expression of FOXO1 in the MDA-MB-231 cell line and to explore the target effects of FOXO1 with hsa-microRNA-204-5p (miR-204) on the biologic behavior of MDA-MB-231 cells. The expression of FOXO1 mRNA and protein in MDA-MB-231 cells were derived and verified from the public databases, literature, and experimental assays, then the downregulation of FOXO1 was confirmed in the MDA-MB-231 cell line. The target binding of FOXO1 and miR-204 was predicted by miRWalk and confirmed by luciferase reporter assays. MiR-204 targeted the 3' untranslated region of FOXO1 and reduced FOXO1 expression in miR-204-transfected cells, resulting in cell growth amplification but inhibition of cell migration and apoptosis, which were assessed using the MTT method, wound healing assays, and flow cytometry, respectively. The protein levels of serine-threonine kinase (AKT), c-jun N-terminal kinase (JNK), extracellular regulatory protein kinase (ERK), and the phosphorylated protein kinases (P-AKT, P-JNK, and P-ERK) were measured by western blot. It was found that AKT, JNK, and ERK remained constant, but P-AKT, P-JNK, and P-ERK were upregulated after miR-204 transfection. In summary, the expression of FOXO1 was downregulated in MDA-MB-231 cells; and the target binding of miR-204 and FOXO1 affected phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) signal pathways, leading to different alterations of cellular activity in MDA-MB-231 cells.

Keywords: FOXO1, miR-204, MDA-MB-231, biological behavior

Introduction

As the most frequently diagnosed cancer worldwide among urban females [1, 2], breast cancer (BC) is also a significant threat to women in China at an annual incidence rate of 3.5% [3]. Despite incredible advances in screening methods and treatment strategies that have improved the survival time of BC patients [4-7], the cure rate and five-year survival rate are still major challenges on account of the elusive pathogenesis, insufficient early screening means, deficient therapeutic methods, complex phenotypes, and high recurrence rates [8-13]. Thus, in-depth research on the molecular mechanisms of BC is urgently needed.

FOXO1, also known as FKHR, FKH1, or FOXO1A, is located on 13q14.11 and is assigned to the Forkhead family of transcription factors involved in cellular progression, such as cell cycle regulation and differentiation [14]. Plenty of

research has demonstrated that FOXO1 is regulated by phosphatidylinositol 3-kinase (PI3K)/serine-threonine kinase (AKT) and mitogen-activated protein kinase (MAPK) signal pathways [15-17], which are also activated to modulate the proliferation, migration, and invasion of BC cells [18, 19]. In addition, the effects of FOXO1 in cellular processes and tumor behaviors are also modulated by other small molecules [20].

One significant class of small regulatory molecules is microRNAs (miRNAs) [21-23]. As a type of endogenous non-coding RNA with regulatory function in eukaryotes, miRNAs completely or incompletely bind to the 3' untranslated region (3'-UTR) of FOXO1, then modulate FOXO1 expression through transcription inhibition or direct degradation, and affect cell differentiation, proliferation, apoptosis, and other biologic processes [20, 24, 25]. Hsa-microRNA-204-5p (also known as miR-204, or miR-204-5p) is an

arresting miRNA in tumorigenesis research and has been supposed to be an oncogene or a tumor suppressor in diverse types of cancer [26], including BC [27, 28], and it draws our attention to analyzing and assessing its target effect toward FOXO1.

Nevertheless, the regulatory mechanisms of the miRNA-FOXO1 networks remain largely unknown. Since cell lines are the easily available in molecular biology research, the biologic characteristics of FOXO1 and miR-204 were mainly observed in MDA-MB-231, a well-established triple-negative BC (TNBC) cell line in this study. The expression of FOXO1 in MDA-MB-231 cells was identified by the public data, real-time quantitative PCR (RT-qPCR), and western blot; the target sequence of FOXO1 with miR-204 was certified by the on-line prediction tool-miR-Walk; the binding stability of FOXO1 with miR-204 was verified through the experimental proofs; and the targeting effects on PI3K/AKT and MAPK signal pathways were also explored and analyzed (**Figure 1A**).

Materials and methods

Data extraction for FOXO1 expression assessment

The expression of FOXO1 mRNA and protein was extracted from public databases up to March 31, 2019. Given the complexity of cancer tissues and the fact that the microarray and sequencing data are affected by a large variety of interstitial components, such as fibers, adipose tissues, blood vessels, lymphatics, and numerous inflammatory cells, BC cell lines are the chief subjects of this study. The quantitative level of FOXO1 in the malignant tumor cell strains was downloaded from the Cancer Cell Line Encyclopedia (CCLE) (<https://portals.broadinstitute.org/ccle>) that provides accurate cellular mRNA expression.

Literature retrieval with key terms "(FOXO1 OR FKHR OR FKH1 OR FOXO1A) AND (breast OR mammary gland)" was performed in PubMed, Wiley Online Library, the Web of Science, and EMBASE for eligible publications that provided the cellular protein level of FOXO1. Admission rules: (1) MDA-MB-231 and at least a normal breast epithelial cell line were explored in the study; (2) the cells were not treated with any intervention before protein extraction; (3) protein expression of FOXO1 was detected by

western blot (WB) or other efficient methods; (4) the protein level of FOXO1 could be extracted and quantified. The area of protein bands was processed and calculated by ImageJ software (NIH, Bethesda, MD).

With the key words "(breast OR mammary gland) AND (cell OR cellular) AND (line OR strain)", the gene-chip data containing BC cell lines and immortalized mammary epithelial cell lines in the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) were collected. The enrollment criteria for the GEO data were as follows: (1) the microarray chip contained MDA-MB-231 and at least an immortalized mammary epithelial cell line; (2) the detection of cell lines was repeated twice or more; (3) the cell lines did not receive any intervention. Exclusion criteria included the following: (1) the chip lacked the normal mammary epithelial cell line as a control group; (2) the cell lines were treated by drug treatment or genetic engineering or other interventions. The relative expression of FOXO1 mRNA in BC cell lines and breast epithelial cells was extracted and calculated; simultaneously, the mean value, standard deviation (SD), t/F value, and *P* value were determined for in-depth analysis.

Cell culture

The human embryonic kidney HEK293T cell line and the human MDA-MB-231 cell line were obtained from the Shanghai Institute of Cell Biology (Shanghai, China) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Wisent, Nanjing, China) with 15% fetal bovine serum (FBS; ExCell Bio, Shanghai, China), 1 mM-glutamine and 1 mM penicillin-streptomycin solution at 37°C in a humidified atmosphere consisting of 5% CO₂.

Target prediction

MiRWalk 2.0 (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>) is a widely used online search tool that supplies the largest available collection of predicted and experimentally verified miRNA-target interactions. Besides miR-Walk itself, this online biological database integrates 11 other existing prediction programs, including miRanda, miRDB, MicroT4, miRMap, miRNAMap, miRBridge, PITA, PICTAR2, RNAhybrid, RNA22, and TargetScan. The complementary sequences between FOXO1 3'UTR and miR-204 was built with the help of this database.

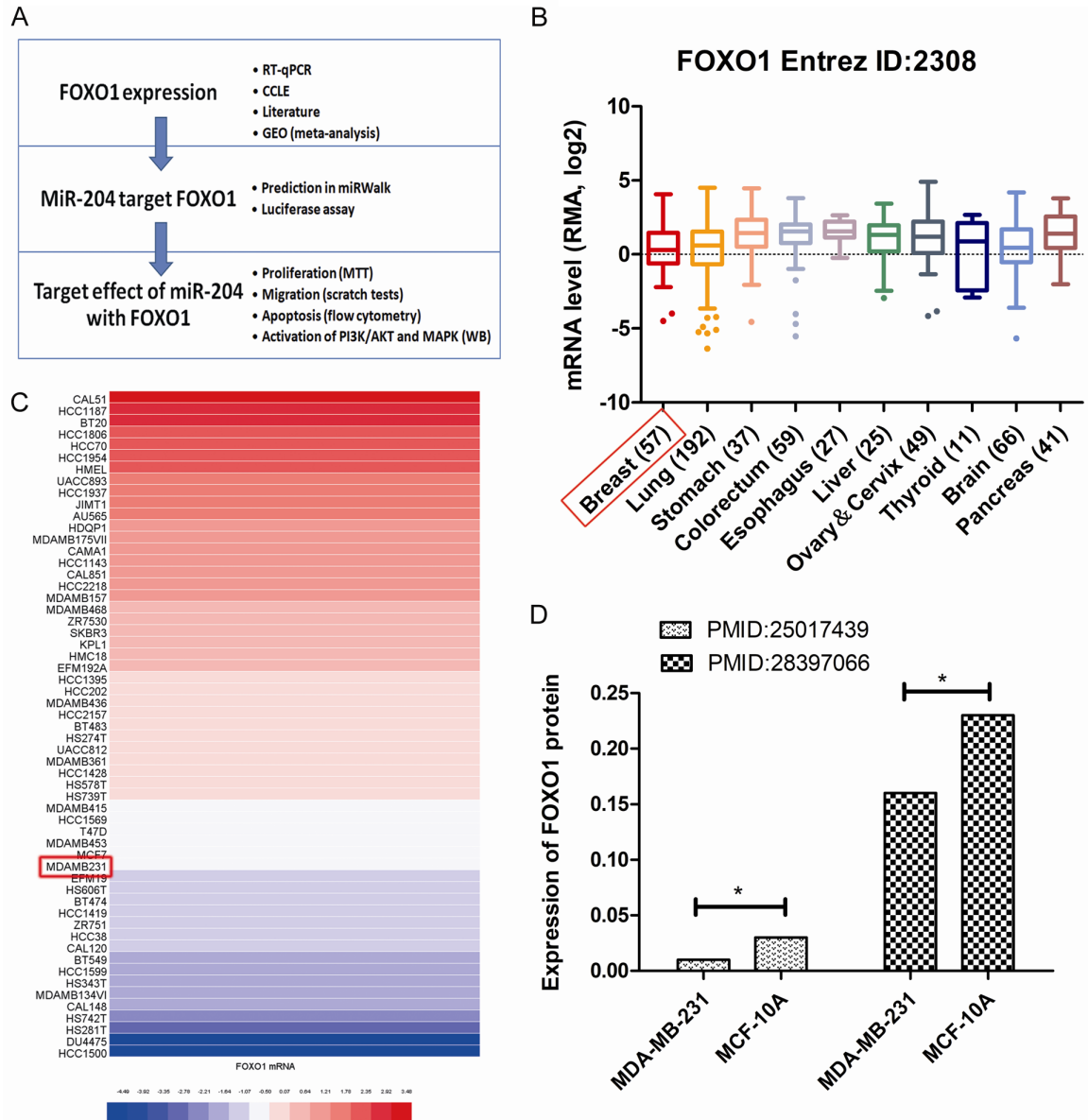


Figure 1. A. Chief research contents and methods in this study. Notes: RT-qPCR, Real-time quantitative PCR; CCLE, the Cancer Cell Line Encyclopedia; GEO, the Gene Expression Omnibus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI3K/AKT, phosphatidylinositol 3-kinase/serine-threonine kinase; MAPK, mitogen-activated protein kinase; WB, western blot. B. Data from CCLE indicated that the FOXO1 mRNA was reduced in the breast cancer cell lines, $F = 45.483$, $P = 0.000$. C. CCLE indicated that the decrease of FOXO1 mRNA was more evident in MDA-MB-231 than other common BC cell lines like MDA-MB-468, T47D, and MCF-7. D. The protein level of FOXO1 in MDA-MB-231 cells was extracted from the eligible articles and the stabilized diminution of FOXO1 protein was observed, $*P < 0.05$.

Luciferase assay

The dual luciferase gene reporter vectors were designed and synthesized by Genechem Biotech (Shanghai, China), and the experimental process was implemented according to the manufacturer's instructions. In brief, HEK293T cells were cultured in 24-well plates at a density of 1.5×10^5 cells/well for 24 h and were co-

transfected by the vectors using Lipofectamine 2000 (Invitrogen, USA). Luciferase activity was determined by the Dual Luciferase Reporter Assay System (Promega, USA).

RNA extraction and detection

The total RNA of MDA-MB-231 cells in the experimental groups was isolated and extract-

Table 1. Primer sequences used in real-time quantitative PCR

Item	Sequence (5'-3')
miR-204	TCCCTTTGTATCCTATGCCT
U6	GCTTCGGCAGCACATATACTAAAAT
FOXO1 forward	CTTGGAGAAGGGGATGTGC
FOXO1 reverse	TGTTGGTGATGAGAGAAGGTTG
β -actin forward	GCACCACACCTTCTACAATGAGC
β -actin reverse	GGATAGCACAGCCTGGTAGCAAC

ed following the standard protocol of AxyPrep Multisource Total RNA Miniprep kit (Axygen, Suzhou, China). The concentration of total RNA was identified by a NanoDrop 2000 instrument (Thermo Fisher Scientific, Waltham, MA, USA). U6 and β -actin were used as housekeeping genes. The primers of miR-204 and U6 were provided by TianGen (Beijing, China) and the primers of FOXO1 and β -actin were procured from Sangon (Shanghai, China). The primer sequences are listed in **Table 1**. RT-qPCR was respectively executed on SYBR-Green Premix Ex Taq (Roche Life Science) and miRcute miRNA qPCR detection kits (SYBR-Green) (TianGen). Then, the relative expression of FOXO1 and miR-204 were calculated and quantified using the comparative $2^{-\Delta\Delta Ct}$ method.

MiRNA vector construction and transfection

The lentiviral vectors of miR-204 (LV-mir204) and the corresponding negative control (LV-mir204-NC) were designed and synthesized by Genechem Biotech (Shanghai, China). Cells in the exponential growth stage were transfected with LV-mir204 or LV-mir204-NC following the manufacturer's instructions, were seeded and incubated in a six-well plate at the density of 5×10^5 /well and then were harvested after three days of transfection. The transfection efficiency of lentiviruses was analyzed by a fluorescence microscope and RT-qPCR.

The experiment was performed with three groups: a control group (MDA-MB-231 cells without any intervention), an NC group (MDA-MB-231 cells infected with the LV-mir204-NC), and an LV-mir204 group (MDA-MB-231 cells infected with the LV-mir204).

Cell proliferation assay

Cells from the three groups were separately grown in 96-well plates at a density of 3,000

cells/well for 4 h, were then maintained for additional 24 h, 48 h, 72 h, and 96 h respectively after the medium replacement with 2% FBS and 100 μ L of culture solution contained 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA). Subsequently, MTT was dissolved by dimethyl sulfoxide (DMSO; Sigma-Aldrich) and the optical density (OD) value was measured by a microplate reader (Scientific Multiskan FC; Thermo Fisher Scientific) at a wavelength of 490 nm.

Cell migration test

MDA-MB-231 cells that were untreated or treated with vectors were seeded in six-well plates and retained until approximately 90% confluence. Each cell monolayer was scratched to create two linear regions that were devoid of cells with a 20 μ L pipette tip then rinsed with phosphate buffer solution (PBS) three times to obliterate the cellular debris. Each study sample was in continued incubation with fresh DMEM containing 2% FBS. Wound closure images in each well were captured by an inverted microscope at 0 h, 24 h, 48 h, 72 h, and 96 h post-scratching. The cell motility was assessed and qualified by the scratch width comparison of each group using ImageJ (NIH, Bethesda, MD). Five random fields in each well were selected and examined three times independently.

Cell cycle and apoptosis assay

The transfected cells and the control group were synchronized with FBS-free DMEM prior to digestion and collection. Then, they were washed with pre-cooling PBS and fixed overnight in 70% ethanol at 4°C. The immobilized cells that were preprocessed with an additional PBS washout were incubated with RNase A (Sigma, CA, USA) and stained with propidium iodide (PI; Sigma, CA, USA) for 30 min at room temperature in dark. The relative content of DNA was estimated by a FACScalibur flow cytometry (BD Biosciences, USA) and the cell cycle distribution was determined by FACSDiva Software.

The processed MDA-MB-231 cells were collected after trypsinization, subsequently washed with cold PBS twice, and resuspended at a density of 1×10^6 /ml in 1 \times Binding Buffer. The cell suspensions were incubated and stained with

FOXO1 and miR-204-5p affect MDA-MB-231

Table 2. Detailed information of the datasets from the Gene Expression Omnibus database

GSE ID	Main contributor	Nation	Year	Platform	MDA-MB-231	MCF-10A	t value	P value	Citation
					n	n			
GSE10890	Alex T.	USA	2008	GPL570	4	2	-39.062	0.000	[56]
GSE41445	Groth P	Germany	2012	GPL570	3	3	-10.818	0.000	[57]
GSE48398	Bryan BA	USA	2013	GPL10558	6	3	-41.217	0.000	[58]
GSE59228	Dupont S	Italy	2014	GPL570	4	4	-14.718	0.000	[59]
GSE59732	Horton JK	USA	2014	GPL571	3	3	-7.760	0.001	[60]
GSE87669	Dobson J	USA	2016	GPL6244	3	2	-10.266	0.002	[61]

n, repetitions of mRNA profile on the cell lines.

mixture of PE Annexin V (5 μ L) and 7-AAD (5 μ L) for 15 min at room temperature away from light and then sufficiently blended with 400 μ L 1 \times Binding Buffer prior to the apoptosis rate detection by a FACScalibur flow cytometer (BD Biosciences, USA).

Western blot

MDA-MB-231 cells in each group were harvested and lysed in RIPA buffer (Beyotime, China) after planting or transfection. The protein extraction and concentration were conducted using a BCA assay kit (Beyotime, China) per the manufacturer's protocol, and the detailed process was completed as previously described [29]. The antibodies of FOXO1 and GADPH were purchased from Proteintech, the antibodies of AKT, c-jun N-terminal kinase (JNK), extracellular regulatory protein kinase (ERK), and the phosphorylated protein kinases (P-AKT, P-JNK, and P-ERK) were provided by Cell Signaling Technology. GADPH was used for protein normalization. The results were analyzed and quantified by ImageJ.

Statistical analysis

All results in this study were derived from at least three independent experiments and reported as mean \pm SD or percentile. The differences between two groups were analyzed using a two-sided Student's t-test. Datasets with three groups or more were compared by one-way analysis of variance (ANOVA). All statistical calculations were treated using SPSS v22.0 (SPSS Inc., Chicago, IL, USA), and $P < 0.05$ was the cut-off value that indicated significance.

Meta-analysis was operated on the Stata 12.0 (Stata Corp LP, College Station, USA) to obtain the standard mean deviation (SMD), 95% confidence interval (95% CI), and P value for the

assessment of the GEO results. The analytical methods were identical to those in previous studies [30, 31], $P < 0.05$ was also considered the threshold value that indicated significance.

Results

The steady downregulation of FOXO1 is found in MDA-MB-231 cells

From CCLE, the expression of FOXO1 mRNA was lower in BC cell lines than other strains ($F = 45.483$, $P = 0.000$), which were cellular-level research alternatives of the female cancers with leading incidence in China [3] (**Figure 1B**). The decrease of FOXO1 mRNA was more evident in MDA-MB-231 than in other frequently-used BC cell lines, such as MDA-MB-468, T47D, and MCF-7 (**Figure 1C**).

A total of 122 articles were obtained from preliminary information retrieval, but only two of these (PMID: 25017439 [24] and PMID: 28397066 [32]) were eligible for further research. The decrease of FOXO1 protein in MDA-MB-231 was consistent with mRNA and was more obvious than in the immortalized mammary epithelial cell line, MCF-10A (**Figure 1D**).

Given that MDA-MB-231 and MCF-10A account for the majority of BC studies [33], data collection in GEO mainly focused on the two cell lines. A total of six datasets that met the enrollment criteria were extracted and their detailed information is listed in **Table 2**. The histograms in **Figure 2A** indicated that FOXO1 mRNA were more evident in MDA-MB-231 than in MCF-10A. Meta-analysis confirmed the downregulation of FOXO1 mRNA was markedly in MDA-MB-231 (SMD = -11.333, 95% CI = -16.391 ~ -6.276, $P = 0.000$) (**Figure 2B**). Random models were applied due to the great heterogeneity among these studies ($I^2 = 58.8\%$, $P = 0.033$).

FOXO1 and miR-204-5p affect MDA-MB-231

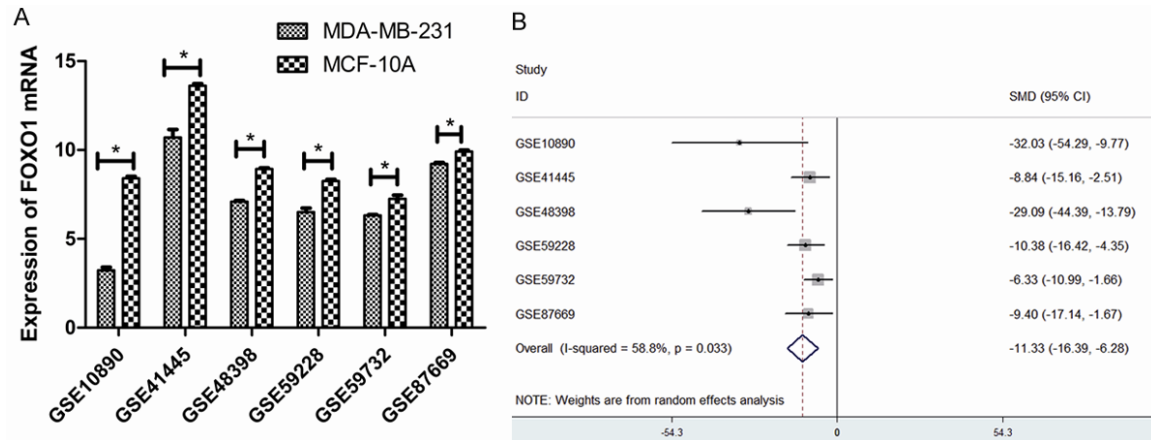


Figure 2. A. FOXO1 mRNA was lower in MDA-MB-231 than in MCF-10A from the datasets of the Gene Expression Omnibus database (GEO), * $P < 0.05$. B. FOXO1 was markedly decreased in the MDA-MB-231 cell line compared to MCF-10A (the standard mean deviation (SMD) = -11.333, 95% confidence interval (CI) = -16.391~-6.276, $P = 0.000$).

FOXO1 is directly targeted by miR-204

Sequence analyses from the prediction tools in miRWalk revealed that 3'-UTR of FOXO1 was highly complementary with a conserved binding site of miR-204 (Figure 3A). The subsequent luciferase reporter gene assays indicated that miR-204 overexpression was notably impaired the luciferase activity of HEK293T cells that were co-transfected with wild-type FOXO1 3'-UTR, but the suppressive effects were abolished in the cells transfected with the mutant vectors (Figure 3B). The results implied that FOXO1 was suppressed by miR-204 at a post-transcriptional level through specific target binding.

MiR-204 promotes proliferation but inhibits migration and apoptosis in MDA-MB-231 cells

The transfection effects of miR-204 lentiviral vectors were first checked by a fluorescence microscope. As shown in Figure 3C, bright green fluorescence appeared in MDA-MB-231 cells treated with LV-mir204 and LV-mir204-NC, but no emissive light was observed in the control group. The RT-qPCR results further demonstrated that the level of miR-204 mRNA was sharply increased in the LV-mir204 group but was nearly unchanged in the NC and control groups (Figure 3D). The above outcomes indicated the high transfection efficiency of lentiviral vectors in the MDA-MB-231 cell line and

provided an ideal foundation for intense research.

The MTT assay was performed at 24 h, 48 h, 72 h, and 96 h after transfection to pinpoint the impact of miR-204 on cell proliferation. The differences in cell growth among the three groups started at 48 h, and the significance between the LV-mir204 group and the control group arose at 96 h, but no obvious significant difference in the proliferation ability of MDA-MB-231 cells was determined between the NC group and the control group at all the observation time points (Figure 3E), suggesting that miR-204 overexpression accelerated MDA-MB-231 cell multiplication in a time-dependent pattern.

The wound healing assays were conducted to appraise the motility of MDA-MB-231 cells and revealed that the LV-mir204 group exhibited a significantly weak migratory ability of cells in comparison to the NC group at 72 h ($P < 0.001$), but the cell migration in the control group and the NC group was unaffected at any checkpoint ($P > 0.05$) (Figure 3F, 3G).

The apoptotic rate of MDA-MB-231 cells was determined by the flow cytometry and was respectively $27.27\% \pm 1.15\%$ in the LV-mir204 group, $31.07\% \pm 1.72\%$ in the NC group, and $36.17\% \pm 0.91\%$ in the control group, illustrating a significant difference in the LV-mir204 group in comparison to the other groups ($F =$

FOXO1 and miR-204-5p affect MDA-MB-231

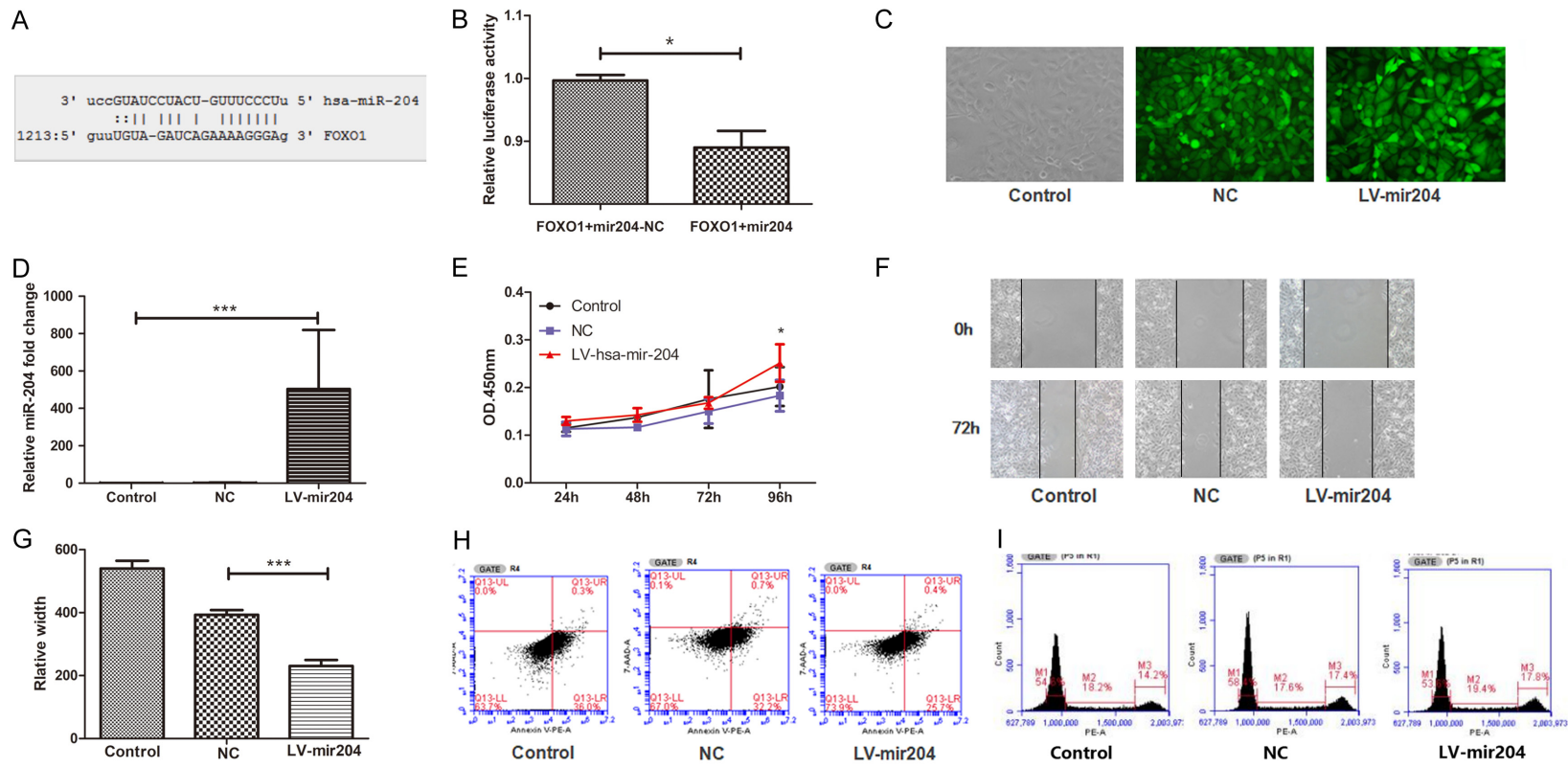


Figure 3. A. Predicted target sequences between miR-204 and FOXO1. B. Luciferase assay of HEK293T cells transfected with miR-204; *P < 0.05. C, D. The transfection efficiency assessed by fluorescence detection techniques and RT-qPCR, respectively; ***P < 0.001. E. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays revealed the growth of MDA-MB-231 cells transfected with miR-204 was accelerated; *P < 0.05. F, G. The wound healing assays suggested that the suppressive effects of miR-204 on cell migration was significant at 72 h; ***P < 0.001. H, I. Flow cytometric analysis confirmed miR-204 reduced the apoptotic rate of MDA-MB-231 cells and arrested the cell cycle at the G2/M phase.

FOXO1 and miR-204-5p affect MDA-MB-231

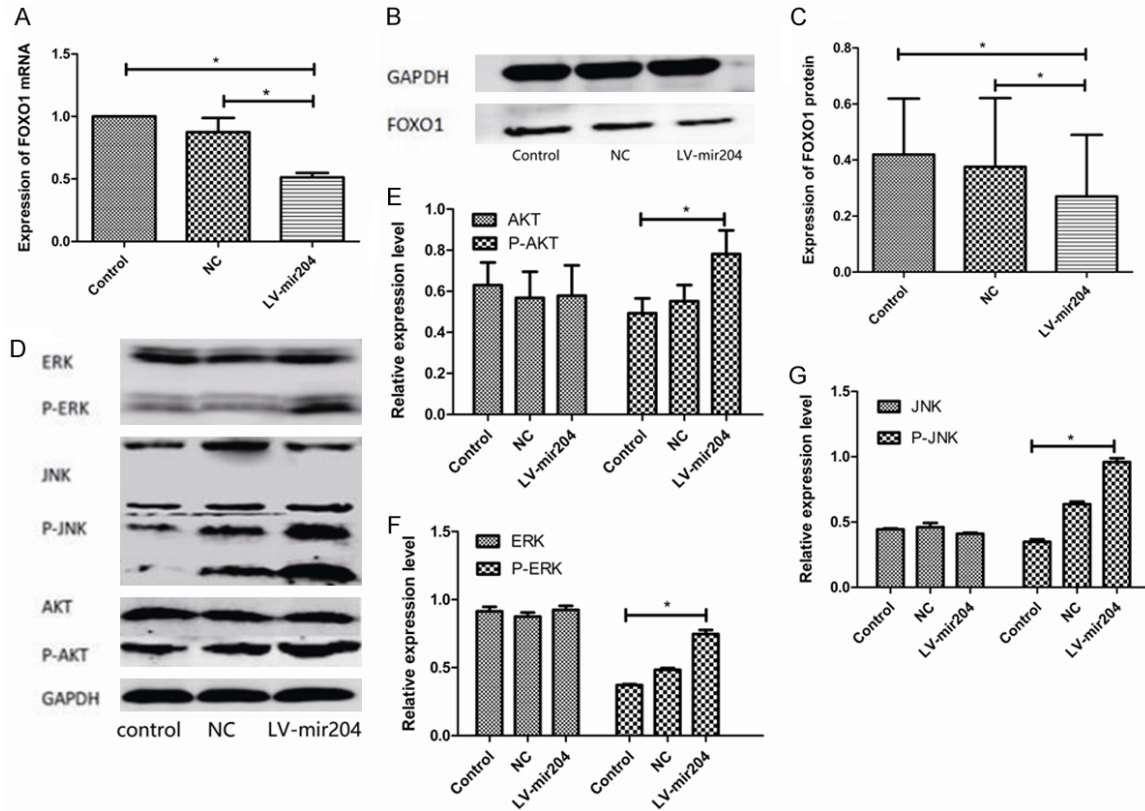


Figure 4. A. The FOXO1 mRNA was decreased after miR-204 transfection; * $P < 0.05$. B, C. The FOXO1 protein was downregulated by miR-204 and was consistent with mRNA expression; * $P < 0.05$. D-G. The target binding of miR-204 with FOXO1 affected AKT, extracellular regulatory protein kinase (ERK), and c-jun N-terminal kinase (JNK) by phosphorylation; * $P < 0.05$.

35.263, $P < 0.01$) (**Figure 3H**). The results indicated that miR-204 is an inhibitory constituent on cell apoptosis.

The cell cycle distribution detected by the flow cytometry revealed that the MDA-MB-231 cells contained the LV-mir204 vectors presented an elevated percentage (17.8%) in the G2/M phase and their apoptotic resistance was affected to some extent, although the comparison of cell counts among the three groups did not achieve statistical significance ($P > 0.05$) (**Figure 3I**).

FOXO1 is downregulated by miR-204 in MDA-MB-231 cells

The assessment of FOXO1 mRNA and protein in the LV-mir204, NC, and control groups was measured by RT-qPCR and WB respectively. The FOXO1 mRNA was strikingly reduced in LV-mir204-transfected MDA-MB-231 cells more than in the two other groups ($P < 0.05$), but its expression in the NC group and the control group demonstrated no significant change ($P >$

0.05) (**Figure 4A**). The variation of FOXO1 protein was consistent with the mRNA level in the three groups (**Figure 4B, 4C**), reconfirming a negative correlation between FOXO1 and miR-204.

MiR-204 activates key proteins in the PI3K/AKT and MAPK signaling pathways

The results of WB demonstrated no significant change of the total amount of AKT, ERK, and JNK in MDA-MB-231 cells after miR-204 over-expression (**Figure 4D**), while the protein levels of P-AKT, P-JNK, and P-ERK in the LV-mir204 group were increased in comparison to those in the NC and control groups ($P < 0.05$) (**Figure 4E-G**), indicating that miR-204 affected the PI3K/AKT and MAPK pathways in the MDA-MB-231 cell line.

Discussion

In the current study, the downregulation of FOXO1 in the MDA-MB-231 cell line was ana-

lyzed and determined by experimental assays and public database mining; FOXO1 was suppressed by direct target binding with miR-204; the targeting effects of FOXO1 and miR-204 affected the PI3K/AKT and MAPK pathways, leading to the regulation of proliferation, migration, and apoptosis in MDA-MB-231 cells and providing a new direction for the molecular mechanism research of BC.

FOXO1 is a critical negative tumor transcription factor and functions in numerous cell biological events, including cell growth, cell cycle regulation, apoptosis, autophagy, DNA damage repair, stress tolerance, and tumorigenesis [17]. Previous studies indicate that FOXO1 is a downstream substrate of the PI3K/AKT signaling pathway. The activated PI3K/AKT pathway mediates FOXO1 protein transportation from the cell nucleus to cytoplasm, creates FOXO1 degradation or transcriptional activation deficiency via dephosphorylation, then excites the transcription of FOXO1 target genes, such as PUMA, Bim, FasL, GADD45, p21 (Cip1), p27 (Kip1), and Cyclin D1/2, which finally causes decreased apoptosis, cell cycle arrest, and impairment in DNA repair processes [34, 35]. AKT is a protein with a molecular weight of 60kD and is the main downstream target of PI3K, conveying important information of PI3K and acting as the central link of the PI3K/AKT signaling pathway [36]. P-AKT is a marker of functional activation of the kinase and stimulates the phosphorylation of other proteins, such as FOXO1 [20, 36], resulting in the accelerated proliferation [37] and apoptosis resistance of BC cells [38].

MAPK activity is essential in the control of the BC process, affects the proliferation, apoptosis, migration, and invasive ability of tumor cells, and also regulates the cell cycle and angiogenesis [19]. ERK and JNK are characterized as two important parallel branches of the MAPK signaling pathway, and their phosphorylation is a sign of MAPK activation [19, 39]. P-ERK reduces FOXO1 activation and then regulates autophagy in tumor cells [40]. Researchers claimed that low expression of FOXO1 is associated with active P-ERK and tumor progression, and even induces drug resistance; while the AKT-phosphorylated FOXO1 binds to IQGAP1 and inhibits ERK phosphorylation in cancer cell lines [41]. FOXO1 has been certified to be a key site for the AKT-ERK cascade [16,

42-44] and participates in epithelial-stromal transformation (EMT) [43, 45]. The JNK pathway is also considered a vital target for the regulation of health and disease status [46] and is closely related to the prognosis and chemoresistance of TNBC [47]. The expression of P-JNK and FOXO1 shows a negative correlation in gastric cancer and JNK activation indicates a poor prognostic implication [48]. FOXO1 directly acts on JNK and inhibits its activation to reduce the transcription levels of p27 (Kip1), Bim, and GADD45; moreover, the FOXO1 transcriptional activity also affects the phosphorylation of AKT and accelerates the repair of cellular DNA damage [49]. The above evidence confirms that FOXO1 is an important nexus between PI3K/AKT signal transduction and the MAPK pathway. MDA-MB-231 is an aggressive TNBC cell line that accounts for approximately two-thirds of TNBC in vitro studies [33], its adjoint presence of FOXO1 downregulation was identified in our study. The existing results indicated that the lack of FOXO1 in MDA-MB-231 cells affects the expression of upstream and downstream targets, then leads to cell function disorders modulated by PI3K/AKT and MAPK signaling pathways and other relevant genes.

MiRNA is an important regulatory factor of gene expression in a variety of ways from transcriptional regulation to post-translational protein modification, and plays a dual role of oncogene or tumor suppressor in BC progression [50]. Several miRNAs have been verified as regulators of FOXO1 in diverse cancer types [14, 20]. MiRNAs maintain mitochondrial homeostasis and regulate associated pathologies by the combined action with FOXO1 [51], they modify FOXO1 by phosphorylation, acetylation, methylation, and ubiquitination, affecting the metabolism, proliferation, invasion, and apoptosis of tumor cells [20]. MiR-9 directly binds to the 3'UTR of FOXO1, leading to decreased transcription and translation of FOXO1 and enhanced proliferation, migration, and invasion of BC cells [32]. In another study, FOXO1 3'UTR was reported to be a promising miRNA-suppressant by controlling the activity of miR-9 [24]. FOXO1 mediates the regulation of miR-544 on the proliferation, migration, and cell cycle distribution of colon cancer cells [25]. The proliferation of BC cells is enhanced when the target binding of miR-223 with FOXO1 takes effect [52]. MiR-222 induces drug resistance in

MCF-7 cells by targeting FOXO1 and activating the PI3K/AKT signaling pathway [53].

As a momentous mRNA regulator, miR-204 is in human chromosome 9 (from 70809975 to 70810084) and originates from the sixth intron of TRPM3, which is abnormally expressed in tumors and closely related to cell proliferation, invasion, metastasis, drug resistance, and poor prognosis [26]. Mounting evidence suggests that miR-204 generally functions on target genes by binding to the 3'-UTR. MiR-204 targets AREG to reduce AKT activity and reverse cell proliferation mediated by the AKT pathway in BC [54]. After target combination with PIK3CB (an important regulatory gene of the PI3K/AKT signal pathway), miR-204 regulates cytokine expression, reprograms immune microenvironment, and enhances the growth, proliferation, migration, and chemotherapeutic response of MDA-MB-231 cells [28]. A recent report revealed that miR-204 also mediates MAPK, WNT, Hedgehog, p53, and TGF-signaling pathways to adjust the self-renewal and EMT ability of BC stem cells by target binding with STAT3 and FOXC1 [27]. In addition, miR-204 attenuates angiogenesis by reducing the protein expression and phosphorylation of target genes involved in PI3K/AKT, RAF1/MAPK, VEGF, and FAK/SRC signaling pathways [55]. Our results suggested that FOXO1 was a target gene of miR-204, and the target binding of the both affected AKT, ERK, and JNK in PI3K/AKT and MAPK signaling pathways, leading to enhanced proliferation, restrained migration, and decreased apoptosis in MDA-MB-231 cells. According to the above references, the possible mechanisms were deemed that miR-204 anchored in FOXO1 3'-UTR and impaired its protein synthesis by modulating DNA transcription and RNA translation; the target binding of FOXO1 with miR-204 affected the PI3K/AKT and MAPK signaling pathways, which influenced FOXO1 transport and degradation and then regulated the biological behaviors of tumor cells.

However, the current research was executed to mainly investigate the FOXO1 expression and the target activity of FOXO1 with miR-204 on the PI3K/AKT and MAPK pathways at the cellular level, which was limited by the lack of animal experiments and clinical studies. In addition, the regulatory mechanisms of FOXO1-miRNA network in BC are also affected by multiple factors, which merit in-depth research.

In conclusion, FOXO1 was downregulated in the MDA-MB-231 strain, the target binding of miR-204 with FOXO1 promoted tumor cell proliferation but suppressed migration and apoptosis by affecting the PI3K/AKT and MAPK signaling pathways.

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Disclosure of conflict of interest

None.

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FOXO1 and miR-204-5p affect MDA-MB-231

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