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Original Article

miR-3614-3p suppresses cell aggressiveness of human breast cancer by targeting *AKT3* and *HDAC1* expression

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Background: MicroRNAs (miRNAs) regulate various pathophysiological functions and pathobiological progression in various cancers. Our recent study reported that miR-3614-3p significantly suppressed the proliferation of breast cancer (BC) cells by downregulating its host gene *TRIM25*. However, other functional roles of miR-3614-3p migration and invasion in BC and its potential mechanisms are not clearly elucidated.

Methods: In this study, we investigated miR-3614-3p regulation of *AKT3* and *HDAC1* expression in BC. miR-3614-3p and *AKT3/HDAC1* mRNA expression levels were determined using quantitative real-time PCR (qRT-PCR) in MCF-7 and MDA-MB-231 BC cells. The effects of miR-3614-3p on migration and invasion were measured using wound healing and transwell migration assays. In BC cells, miR-3614-3p levels were remarkably low, and *AKT3* and *HDAC1* mRNA and protein levels were high as assessed by qRT-PCR and western blot. Finally, we investigated the role of *AKT3/HDAC1* using silent interfering RNA (siRNA) and confirmed the targeting of *AKT3* and *HDAC1* 3' UTR through miR-3614-3p using a luciferase reporter assay.

Results: In the present study, we found that overexpression of miR-3614-3p markedly suppressed tumor cell invasion and migration independent of *TRIM25*, whereas other target genes, *AKT3* and *HDAC1*, were involved. Moreover, we found that the resulting silencing of *AKT3* and *HDAC1* suppressed cell migration.

Conclusions: miR-3614-3p is an anti-oncogene that can suppress BC cells by targeting *AKT3* and *HDAC1*, revealing the potential role of miR-3614-3p in suppressing BC metastasis. Therefore, miR-3614 may act as a potential biomarker for BC prognosis.

Keywords: Breast cancer (BC); miR-3614; *AKT3*; *HDAC1*

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Introduction

Breast cancer (BC) is one of the most common types of cancer and is the most prevalent aggressive malignancy in women. BC is treated with a combination of surgery, chemotherapy, radiation therapy, and hormone therapy (1-3). Despite technological advances in treatment, most patients with BC have a poor prognosis due to the high frequency of distant metastasis or tumor recurrence. Thus, it is necessary to further elucidate the molecular mechanisms involved in the process and development of BC, as many unanswered questions remain regarding effective targets of BC and the necessary development of

effective BC therapies.

MicroRNAs (miRNAs) are endogenous non-coding RNAs, typically about 19–24 nucleotides (nt) in length, that regulate gene expression by binding to the 3'-UTR of many target mRNAs. In doing so, miRNAs induce the degradation of RNA and subsequently affect the transcription and translation of mRNA. Many studies have identified that miRNAs play an essential role in many biological processes, including cell invasion, proliferation, migration, and apoptosis. Due to their multifactorial processes, many miRNAs have also been identified in pathology development resulting from aberrant expressions, such as with several forms of cancer, including gastric cancer, hepatocellular carcinoma, leukemia, lung, and liver cancer, suggesting that they may function as either tumor oncogenes or suppressors (4-7).

miR-3614 is an intragenic miRNA located on chromosome 17, and its host gene is tripartite motif-containing 25 (*TRIM25*). Our previous investigation found that miR-3614-3p suppresses *TRIM25* expression by binding to its 3'-UTR and that induced overexpression of miR-3614-3p inhibits the proliferation of BC cells via downregulation of *TRIM25* (8). Notably, another study reported that miR-3614 suppresses inflammatory responses by knocking down the expression of tumor necrosis factor receptor-associated factor 6 (TRAF6) and targeting the TRAF6/MAPK/NF-κB pathways in coronary artery disease (CAD) epicardial adipose tissue (EAT) (9). Although no other studies have reported the role of miR-3614-3p in the pathology of BC, the -5p of this miR gene has been reported to be associated with colon cancer. From this reporting, miR-3614-5p was identified to suppress the proliferation of colon cancer cells through inhibition of the P53 pathway and p38MAPK pathway (10). However, other functional roles of miR-3614 in cancer development and pathology remain to be elucidated. Therefore, we investigated whether miR-3614 overexpression affects the motility of BC cells and whether this effect is achieved by regulating the expression of *TRIM25*. We present the following article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2419/rc).

Methods

Cell culture

Three cell lines were utilized for this study and purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China): MDA-MB-231, MCF-7, and 293T (HEK) cells. MDA-MB-231 and MCF-7 cells were grown in Falcon culture dishes with Dulbecco Modified Eagle medium (DMEM), which was supplemented with 1% antibiotics and 10% fetal bovine serum (FBS; all from Sigma-Aldrich; St. Louis, MO, USA) in a CO_2-regulated incubator at 95% humidity and 5% CO_2. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Plasmid construction

The miR-3614 expression vector (miR-3614) and the control vector were synthesized and cloned into pcDNA^{TM}6.2-GW/EmGFPmiR plasmids (Invitrogen; Carlsbad, CA, USA). The binding site of miR-3614 in the 3'-UTR of human *AKT3* and *HDAC1* mRNA was constructed and cloned between the XhoI and SacI sites of the pmirGLO dual-luciferase miRNA target expression vector (Promega; Madison WI, USA). We constructed pmirGLO-*AKT3*/*HDAC1* wild-type (WT) and pmirGLO-*AKT3*/*HDAC1* mutant-type (MUT), as described previously (8). Silent interfering RNAs (siRNAs), anti-miR-3614, and their respective negative control RNAs were purchased from Gima (Shanghai, China). The plasmid and miRNA sequences were as follows: miR-3614, GGUU CUGUCUUGGGCCACUUGGAUCUGAAGGCUGCC CCUUUGCUCUCUGGGGUAGCCUUCAGAUCUUG GUGUUUUGAAUUCUUACU, and inhibitor miR-3614-3p: AAAACACCAAGATCTGAAGGCTA. All plasmid and mRNA products were confirmed by sequencing.

Quantitative real-time PCR (qRT-PCR)

According to the manufacturer's instructions, total cellular RNA was extracted using TRIzol reagent (Invitrogen). Real-time PCR was performed using the PrimeScript RT Reagent Kit and SYBR Premix Ex Taq II Kit (Takara, Tokyo, Japan) to detect the expression of mature miR-3614 and *AKT3*/*HDAC1* mRNA, respectively. The relative amount of each miRNA was normalized to U6 small nuclear RNA (snRNA), whereas mRNA expression was normalized to β-actin. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Samples were run using an IQ5 Multicolor qRT-PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Forward and reverse primers were used as follows: *AKT3*, GCAGAGGCAAGAAGAGGAGA and ACTTGCCTTCTCTCGAACCA; HDAC1, CATCTCCTCAGCATTGGCTT and

TATTATGGACAAGGCCACCC.

Western blot analysis

BC cells were harvested using RIPA lysis buffer (CST, Boston, MA, USA) 48 h after transfection. Equal amounts of protein lysates were separated by 10% SDS-PAGE and transferred onto a methanol-activated PVDF membrane (MilliporeSigma; Burlington, MA, USA). The membranes were incubated with the following primary antibodies: *AKT3* (1:2,000; Abcam; Cambridge, UK) and *HDAC1* (1:1,000, Abcam) overnight at 4 ℃. The membranes were incubated with a secondary antibody for 2 h at room temperature, followed by ECL reagent (Millipore, Boston, MA, USA) for chemiluminescence detection, as described previously (8).

Dual-luciferase reporter assay

The *AKT3*/HDAC1-WT or *AKT3*/HDAC1-MUT vector was cotransfected with pre-miR-3614 plasmid or the corresponding control pcDNA6.2-GW into HEK293T cells using 96-well plates. Following a 24 h transfection period, cell lysates were collected and assayed using the luciferase activity assay. According to the manufacturer's protocol, firefly and Renilla luciferase were measured using the Dual-Glo Luciferase Assay System (Promega).

Wound healing assay

To examine the effects of miR-3614 on the migration of BC cells, MCF-7 and MDA-MB-231 cells were plated in 6-well culture plates (1.0×10^6 cells). The cell monolayer was scraped using a P-10 micropipette tip (a line was scratched into the cell monolayer), and the suspension cells were removed following a PBS wash. Subsequently, the cells were transfected with 20 pmol of negative controls, miR-3614 or anti-miR-3614, and further incubated for 48 h. Cells were further incubated following the transfection process to allow cell migration in the reduced serum DMEM media. At 0, 24, and 48 h, three fields of the wound area were imaged with a phase-contrast microscope.

Transwell migration assay

The migration assay was performed using a transwell chamber (MilliporeSigma). After the cells were transfected with 20 pmol of negative controls, miR-3614 or anti-miR-3614, the BC cells were seeded into the upper chambers with serum-free medium (1.0×10^4 cells), and the lower chambers contained DMEM with 10% FBS. After incubation for 24 h, the cells were fixed and stained with crystal violet. Migrated MCF-7 and MDA-MB-231 cells were manually counted under an inverted light microscope.

Statistical analysis

The results are presented as the mean ± SEM of at least three independent experiments. Significance was established using SPSS software (version 22.0; IBM, San Francisco, CA, USA). Student's *t*-test and ANOVA were used if the quantitative data between groups showed a normal distribution. If not consistent with normal distribution, the Wilcoxon-Mann-Whitney test was used. Differences were considered statistically significant at $P<0.05$.

Results

Overexpression of miR-3614-3p inhibits migration and invasion of BC cells

To explore the biological significance of miR-3614-3p in BC, MCF-7 and MDA-MB-231 cells were transfected with miR-3614 plasmid to overexpress miR-3614-3p. Additionally, miR-3614-3p was significantly downregulated by the miR-3614-3p inhibitor. The transfection efficacy was tested by qRT-PCR ($P<0.01$, *Figure 1A*). As shown in *Figure 1B*, wound healing assays revealed that overexpression of miR-3614-3p required a longer time to heal than that of control cells. In addition, the transwell migration assay showed that overexpression of miR-3614-3p dramatically inhibited MCF-7 and MDA-MB-231 cell migration compared to control cells (*Figure 1C*). In contrast, BC cells derived from the miR-3614-3p inhibitor displayed a higher migration ability than the control cells. Collectively, these results demonstrate that miR-3614-3p can manipulate the aggressiveness of BC cells.

Effects of TRIM25 siRNA on BC cell metastasis

Our previous study demonstrated that miR-3614-3p could inhibit BC cell proliferation by targeting *TRIM25* (8). Here, we expand on these studies and use MCF-7 and MDA-MB-231 cells to investigate whether *TRIM25* siRNA can influence tumor cell metastasis, cell migration, and invasive capability. Importantly, si*TRIM25*-transfected cells had similar wound healing rates as the control scrambled-

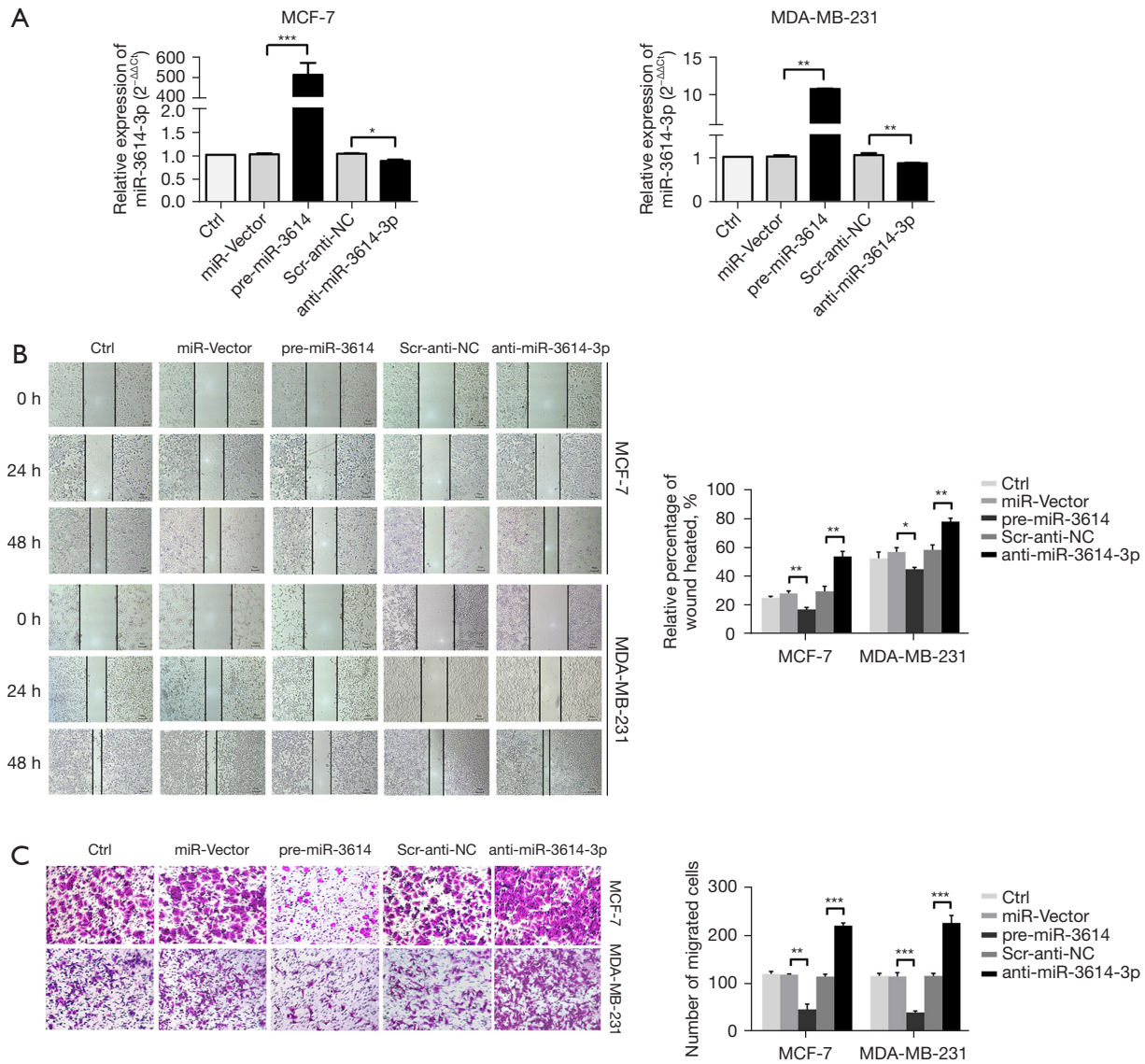

Figure 1 Ectopic expression of miR-3614-3p ameliorates BC migration and invasion *in vitro*. (A) miR-3614-3p was assessed in MCF-7 and MDA-MB-231 cells after transfection with miR-3614 expression vector and anti-miR-3614. (B) Wound-healing assays showed that miR-3614-3p suppressed cell migration. Images were captured at 0, 24, and 48 h after scratching. (C) Transwell assays (magnification ×200) showed that miR-3614-3p suppressed cell metastasis (stained in 0.1% crystal violet for 15 min; upper panel: migration assays; low panel: invasion assays). Scale bars: 100 μm. Experiments were repeated at least 6 times with similar results, and error bars represent ± SD. *, P<0.05; **, P<0.01; ***, P<0.001. BC, breast cancer.

transfected cells (*Figure 2A*, P>0.05); however, compared with control groups, BC cell migration was reduced following transfection with *TRIM25* siRNA (*Figure 2B*, P<0.05). These data suggest that *TRIM25* has no significant effect on the metastasis of BC cells, and there might be other target genes that influence BC cell metastasis.

The mechanism of miRNA-3614-3p self-silence

In addition, we hypothesized that mature miR-3614-3p is cleaved by the Dicer complex and binds to pri-miR-3614, thereby silencing itself through this mechanism. To test this hypothesis, we cloned pri-miR-3614 fragments into

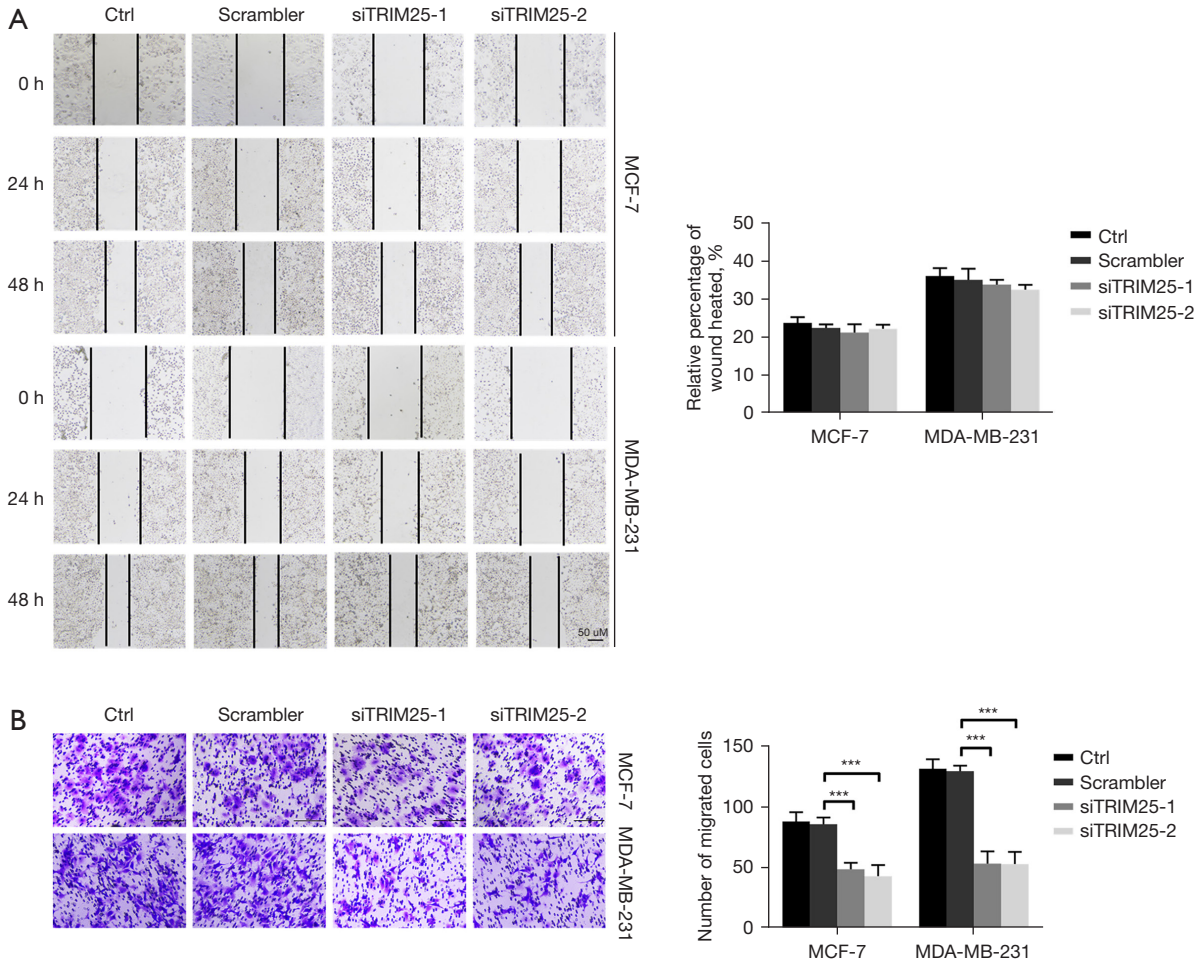

Figure 2 The effect of *TRIM25* knockdown siRNA on BC cell metastasis. (A) Wound-healing assays showed that si*TRIM25* had no obvious difference compared with scrambled controls. (B) Transwell assays (magnification ×200) showed that si*TRIM25* inhibited cell metastasis (stained in 0.1% crystal violet for 15 min; upper panel: migration assays; low panel: invasion assays). Scale bars: 100 μm. Experiments were repeated at least 6 times with similar results, and error bars represent ± SD. ***, P<0.001. siRNA, silent interfering RNA; BC, breast cancer.

the pmiRGLO vector and generated the respective mutants (*Figure 3A*). As expected, the relative luciferase activity was significantly decreased when miR-3614 and pri-miR-3614-pmiRGLO vectors were co-transfected into HEK293T cells, compared with cells transfected with the control pmiRGLO vector. However, we found that the mutation in pri-miR-3614 did not affect the relative luciferase activity. In contrast, transfection with the miR-3614-3p inhibitor enhanced the luciferase activity (*Figure 3B*). Collectively, these results suggest that miR-3614-3p can silence itself.

AKT3 and HDAC1 are direct targets of miR-3614-3p

To elucidate the molecular mechanism by which miR-3614-3p exerts its inhibitory effect on BC cells, we searched for miR-3614-3p target genes using the prediction program TargetScan and found miR-3614-3p binding sites in the UTR of *AKT3* and *HDAC1* 3 (*Figure 4A*). To validate the hypothesis that miR-3614-3p targets *AKT3* and *HDAC1*, a dual-luciferase reporter system containing *AKT3* and *HDAC1* 3 UTR-WT/MUT was used, alongside miR-3614-3p and reporter plasmid or pmirGLO control vectors that were co-transfected into HEK293T cells. As illustrated in *Figure 4B*, miR-3614-3p reduced the firefly luciferase activity of *AKT3* and HDAC1-WT at 24 h compared to the MUT-type and control cells. To confirm whether the loss of miR-3614-3p is a contributing factor in BC malignancy by upregulating *AKT3* and *HDAC1* expression, we performed qRT-PCR and

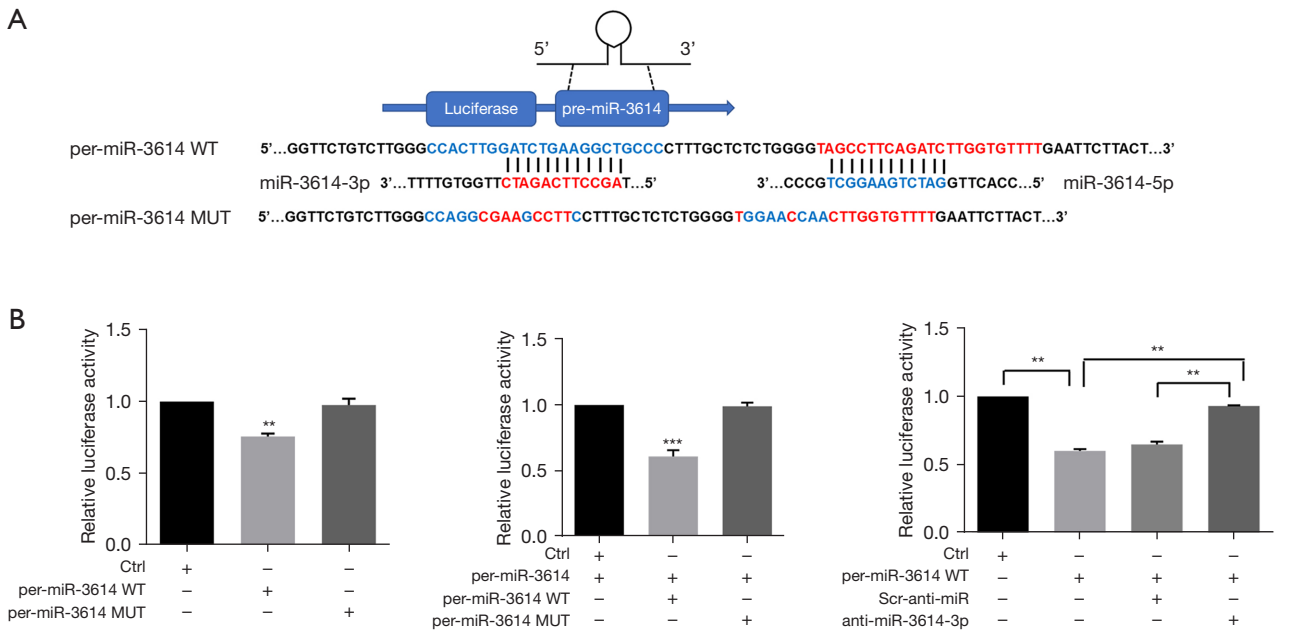

Figure 3 The mechanism of miR-3614 self-silence. (A) miR-3614-3p and miR-3614-5p are highly conserved across species, and they have binding sites within the miR-3614. (B) The luciferase assay was performed in HEK293T cells in which miR-3614 or anti-miR-3614-3p was co-transfected with or without pGLO-miR-3614 wild-type or pGLO-miR-3614 mutant vector. Data are shown as mean ± SEM. **, P<0.01; ***, P<0.001. WT, wild-type; MUT, mutant-type.

western blot analysis. We found that overexpression of miR-3614-3p dramatically decreased the mRNA and protein levels of *AKT3* and *HDAC1* (*Figure 4C*). Moreover, we found that *AKT3* and *HDAC1* levels were higher in BC tissues than in normal tissue controls from the The Cancer Genome Atlas (TCGA) database (P<0.001). Similar results were observed for *AKT3* and *HDAC1* mRNA and protein overexpression in MCF-7 and MDA-MB-231 cell lines, in which miR-3614-3p was downregulated (*Figure 4D*). Taken together, the above data indicate that miR-3614-3p directly regulates *AKT3* and *HDAC1* expression in MCF-7 and MDA-MB-231 cells.

Silencing of AKT3 and HDAC1 reduced BC cells invasiveness

To further validate that miR-3614-3p suppressed BC cell migration by regulating *AKT3* and HDAC1, we inhibited *AKT3* and *HDAC1* mRNA and protein expression by siRNA knockdown (*Figure 5A*). Moreover, silencing *AKT3* and *HDAC1* resulted in the suppression of cell wound healing and migration ability (*Figure 5B,5C*), which followed the same trend as miR-3614-3p in MCF-7 and MDA-MB-231 cells. Based on these findings, *AKT3* and *HDAC1* are essential and appear to serve as functional downstream mediators of miR-3614-3p in BC.

Discussion

Cancer is still predominantly determined by identified alterations in tumor suppressor genes and oncogenes. From our growing knowledge on cancer development and regulation, we understand that these gene expressions can be regulated by miRNAs (11-13). Therefore, researchers focus on addressing two main questions: "How do miRNAs regulate oncogenes and/or tumor suppressor genes?" and "Which miRNAs regulate other mechanisms in cancer cells?" (14). Li *et al.* (15) proposed that phosphoglycerate Mutase 1 (PGAM1), regulated by miR-3614-5p, functions as an oncogene by activating transforming growth factor-β (TGF-β) signaling in the progression of non-small cell lung carcinoma. In addition, Huang *et al.* (9) showed that miRNA-3614 regulates inflammatory response via targeting TRAF6-mediated MAPKs and NF-κB signaling in the EAT with CAD. Our previous study showed that miR-3614-3p was downregulated in BC tumors compared to normal tissues. Moreover, miR-3614-3p expression was reduced in

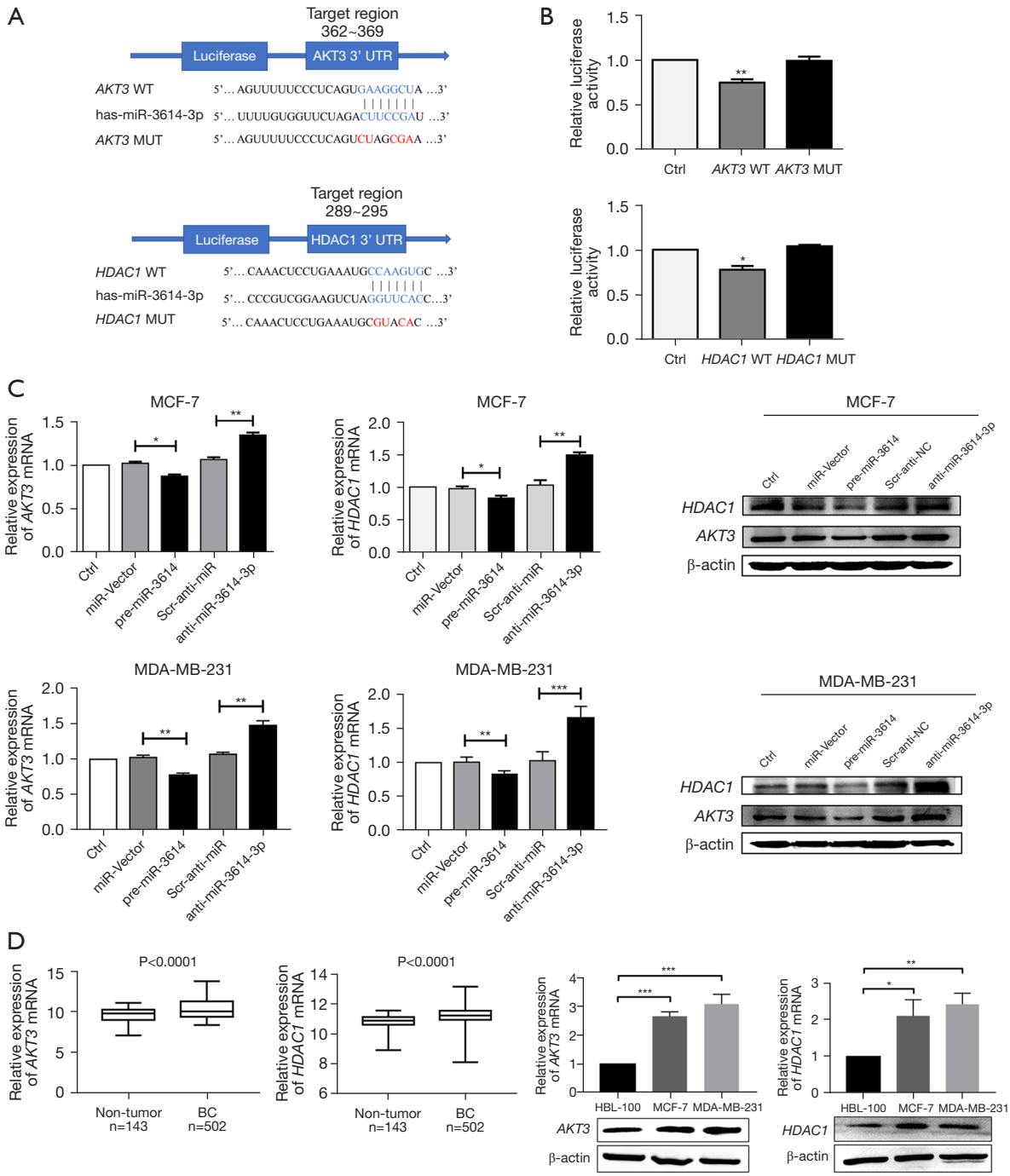

Figure 4 *AKT3* and *HDAC1* are experimentally validated as direct targets of miR-3614-3p in human BC cells. (A) The binding sites of miR-3614-3p at the 3'-UTR of *AKT3* and *HDAC1* were predicted by bioinformatics. (B) Luciferase reporter vectors (including wild or mutant type *AKT3*/HDAC1-3'-UTR) were cotransfected with miR-3614 or Control into HEK293T cells. A dual-luciferase assay examined luciferase activity. (C) *AKT3/HDAC1* mRNA and protein expressions were assessed in MCF-7 and MDA-MB-231 cells after transfection of miR-3614 expression vector/anti-miR-3614. (D) RNA-Seq analysis data of *AKT3* and *HDAC1* expression in BC tissues (n=143) and normal tissues (n=502). RNA-Seq analysis used data download from TCGA. *AKT3/HDAC1* mRNA and protein expressions were assessed in BC cell lines. Data are shown as mean ± SEM. *, P<0.05; **, P<0.01; ***, P<0.001. WT, wild-type; MUT, mutant-type; BC, breast cancer; TCGA, The Cancer Genome Atlas.

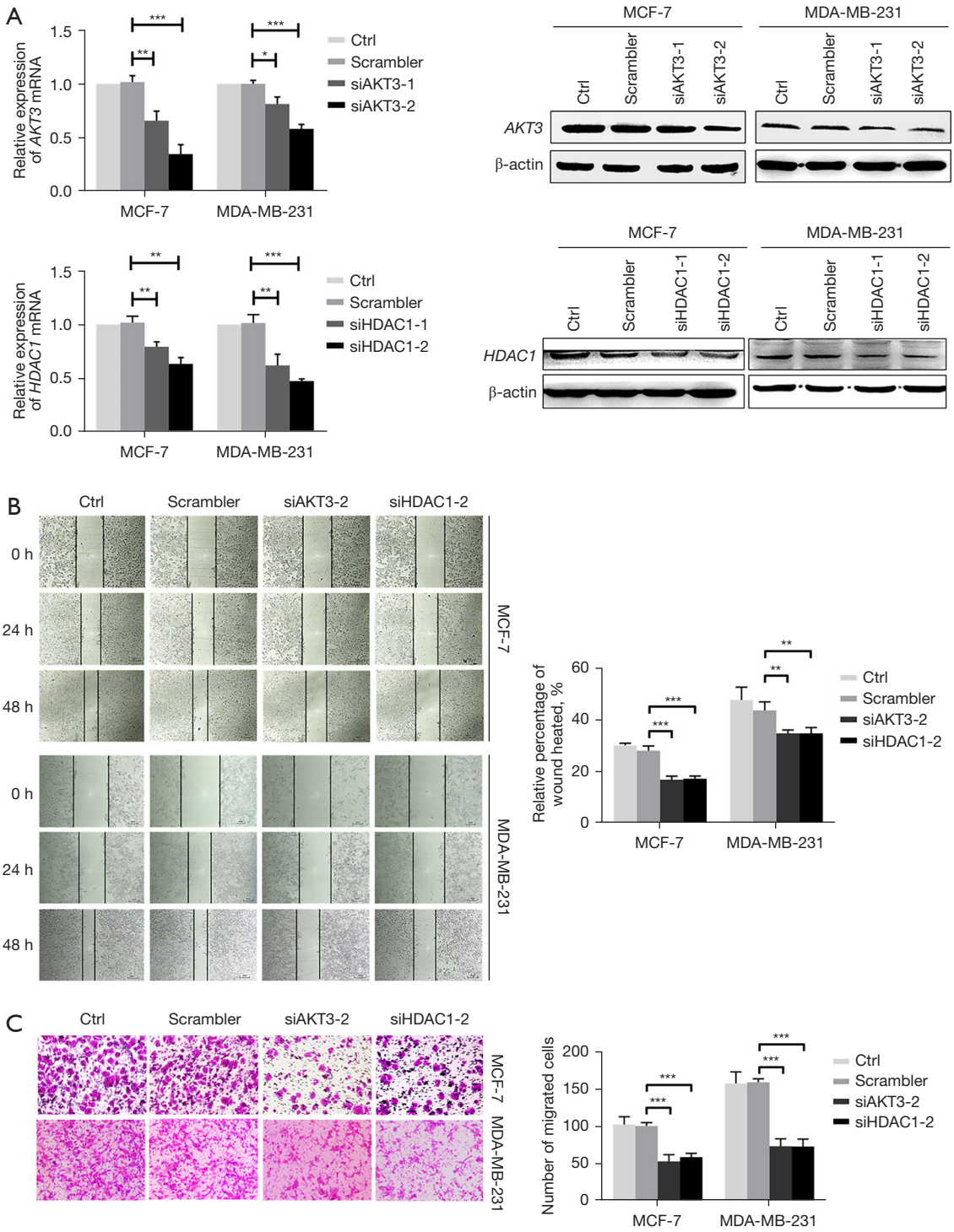

Figure 5 *AKT3* and *HDAC1* are the functional mediators downstream of miR-3614-3p in BC cells. (A) qRT-PCR and western blot were performed to examine the expression of *AKT3* and *HDAC1* after transfection with *AKT3/HDAC1* siRNA. (B) Wound-healing assays showed that si-*AKT3/HDAC1* suppressed cell migration. Images were captured at 0, 24, and 48 h after scratching. (C) Transwell assays (magnification ×200) showed that si-*AKT3/HDAC1* depressed cell metastasis (stained in 0.1% crystal violet for 15 min; upper panel: migration assays; low panel: invasion assays). Scale bars: 100 μm. Experiments were repeated at least 6 times with similar results, and error bars represent ± SD. *, P<0.05; **, P<0.01; ***, P<0.001. qRT-PCR, quantitative real-time PCR; siRNA, silent interfering RNA; BC, breast cancer.

MCF-7 and MDA-MB-231 cell lines compared to HBL-100 epithelial cell lines. In the current study, miR-3614-3p reduced cancer cell invasion and migration abilities. However, the inhibition of invasion and migration was more significant in invasive BC cells (MDA-MB-231) than in non-invasive BC cells (MCF-7) when assessing via transwell matrigel and wound healing assays.

In contrast, anti-miR-3614-3p oligonucleotides were used for our loss-of-function studies to silence the expression of miR-3614-3p. Utilizing this inhibitory effect revealed a significant contribution to the tumorigenicity of MCF-7 and MDA-MB-231 cells. Therefore, these data suggest that miR-3614-3p functions as a tumor suppressor for cell aggressiveness in BC. To this end, added validation using *in vivo* assays is needed for further research.

Additionally, the identification of downstream functional targets is necessary to elucidate the respective biological effects and better comprehend their physiological activity. In our previous study, we found that the overexpression of miR-3614-3p dramatically inhibited BC cell growth through the downregulation of *TRIM25*. However, in this study, we report that *TRIM25* downregulation had no significant effect on the metastasis of BC cells, likely suggesting the possibility of other target genes that are responsible for BC cell metastasis. Using TargetScan, an online prediction software, *AKT3* and *HDAC1* were identified as potential target genes of miR-3614-3p, in which AKT, also known as PKB, is vital for maintaining normal cellular function. Concerning its potential role in cancer, previous studies have identified a correlation between tumor development and *AKT3* expression (16-20). Wang *et al.* (21) verified that miR-384 could significantly suppress the proliferation of colorectal cancer by targeting *AKT3*, and that miRNA-433 targets *AKT3*, thereby inducing the inhibition of cell proliferation and viability in BC (22). In support of these findings, Rui Li and Ying Ma also found that miR-29a and miR-144 can suppress growth and metastasis in papillary thyroid carcinoma and hepatocellular carcinoma by targeting *AKT3* (23).

Histone deacetylases (HDACs) are posttranslational modifiers that deacetylate proteins. Although necessary for vital DNA transcription processes and other metabolic responses, numerous investigations have demonstrated that *HDAC1* can also be involved in tumor progression (24-29). For example, miR-34a regulates *HDAC1* expression and affects the proliferation and apoptosis of hepatocellular carcinoma cells. Likewise, miR-761 has been found to inhibit colorectal cancer cell proliferation and invasion by targeting *HDAC1* (30). He and colleagues also revealed that *HDAC1* promotes migration and invasion by binding with TCF12 and promoting epithelial-to-mesenchymal transition (EMT) progression in gallbladder cancer. Moreover, Liu *et al.* found that the upregulation of *HDAC1* is a crucial event in developing drug resistance to current treatments in ovarian cancer. Thus, targeting *HDAC1* by enhancing c-Myc-dependent miR-34a expression may be an effective strategy for increasing the efficacy of cisplatin treatment (31,32).

To our knowledge, our study is the first to describe that miR-3614-3p directly represses *AKT3* and *HDAC1* expression in BC. From our study, we identified that *AKT3* and *HDAC1* were overexpressed in BC tissues and cell lines. Furthermore, *AKT3* and *HDAC1* are direct downstream targets of miR-3614-3p, and upregulation of miR-3614-3p significantly reduced the expression of *AKT3*/*HDAC1*, thereby decreasing the luciferase reporter activity of *AKT3*/*HDAC1* WT 3-UTR but not MUT 3-UTR. In addition, we showed that silencing *AKT3* and *HDAC1* resulted in the suppression of cell wound healing and migration. Importantly, our previous study showed that *TRIM25* is not only a host gene but also a target gene of miR-3614-3p in BC. This study found that miR-3614-3p significantly binds to pri-miR-3614 at the 3'-UTR of *TRIM25*, suggesting that miR-3614-3p could silence itself. Collectively, these data provide sufficient evidence to support that miR-3614-3p exerts a suppressive effect on BC cells, at least in part, by inhibiting *AKT3* and *HDAC1* expression.

Conclusions

In summary, our results revealed that miR-3614-3p inhibited the migration and invasion capabilities of MCF-7 and MDA-MB-231 cells by targeting *AKT3* and *HDAC1* expression. Thus, we suggest that miR-3614-3p might be an effective biomarker for targeted BC therapies, especially when targeting the invasive form of this disease.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2419/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2419/dss

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2419/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2419/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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