


ORIGINAL ARTICLE

miR-223-3p targets FBXW7 to promote epithelial-mesenchymal transition and metastasis in breast cancer

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Abstract

Background: Breast cancer is the most common malignant tumor diagnosed in women. It is the second leading cause of cancer-related death among women in the world. Aberrant expression of microRNAs (miRNAs) have been identified to be involved in the development and progression of breast cancer. The aim of this study was to investigate the function of miR-223-3p in breast cancer progression and metastasis.

Methods: qRT-PCR was used to analyze the expression levels of miR-223-3p in breast cancer tissues and cell lines. Wound healing and Matrigel assays were used to examine cell motility and invasiveness. FBXW7 3'-UTR construct and luciferase reporter assays were performed for the target gene.

Results: miR-223-3p was overexpressed in breast cancer tissue and cell lines. A high level of miR-223-3p was associated with poor prognosis in breast cancer patients. In addition, overexpressed miR-223-3p promoted the migration and invasion of breast cancer cells in vitro and in vivo. Mechanistically, we found that tumor suppressor gene FBXW7 is a target of miR-223-3p. Luciferase activity reporter assay indicated miR-223-3p could directly bind with the 3'-UTR of FBXW7. miR-223-3p exhibited its oncogenic role partly by decreasing FBXW7 expression, and consequently promoted the invasion and metastasis of breast cancer cells.

Conclusions: Our study revealed a physical and functional relationship among miR-223-3p and FBXW7. By negatively regulating FBXW7 expression, miR-223-3p exerts a tumor promotion role promoting cell invasion and metastasis in breast cancer.

KEYWORDS

breast cancer, epithelial-mesenchymal transition, FBXW7, metastasis, miR-223-3p

INTRODUCTION

Breast cancer is the most common malignant tumor diagnosed in women, accounting for 30% of new cancer diagnoses in women. It is the second leading cause of cancer-related death among women in the world.^{1,2} Surgical resection is the main treatment for breast cancer. However, about 20%–40% of patients will suffer from breast cancer recurrence or metastasis due to the spread of cancer cells.³ Distant metastasis is the leading cause of breast cancer-related death. Great progress has been made in the early diagnosis and treatment of breast cancer, and molecular

targeted therapy and immunotherapy have also become effective treatments for some patients with advanced breast cancer. However, breast cancer occurrence and metastasis is a complex biological process of multifactor and multigene interaction and coregulation. The mechanism of breast cancer metastasis in most patients is still unclear. Therefore, identifying new molecular indicators and therapeutic targets of breast cancer is of great significance to improve the survival of breast cancer patients.

MicroRNAs (miRNAs) are small non-coding RNA molecules which are 18–22 nucleotides in length.⁴ They inhibit the translation or promote the degradation of target genes in

a sequence complementarity-dependent manner by binding to the 3' untranslated region (3'-UTR) of target mRNA.^{5,6} miRNAs can function as oncogenes or tumor suppressors to modulate a variety of physiological process in vivo, including cell cycle progression, stem cell self-renewal, tumorigenesis, immune response, and reshaping the microenvironment.⁷⁻⁹ Mature miRNA duplex contains the guide and lagging strands. In most cases, the lagging strand is less active and does not regulate the expression of target genes while the guide strand is considered as the functional strand which exhibits a miRNA-induced regulatory function.¹⁰⁻¹² miR-223-3p as the guide strand of miR-223 duplex has been recognized as functional in human cancers, including breast cancer, osteosarcoma, neuroblastoma, gastric cancer and liver cancer.¹³⁻¹⁷ Accumulating data indicate that the expression of miR-223-3p is associated with breast cancer progression. Yoshikawa et al. reported that exosomal miR-223-3p was a preoperative biomarker and associated with the malignancy of breast cancer.¹⁷ Du et al. reported that miR-223-3p promoted breast cancer cell proliferation and invasion via the Hippo/Yap signaling pathway.¹⁸ In addition some recent studies also showed that miR-223-3p regulated the occurrence of breast cancer may through targeting multiple genes, such as NLRP3, ECT2 and PFN2.¹⁹⁻²¹ Although there is a preliminary understanding of the function of miR-223-3p in breast cancer, the underlying mechanism that links miR-223-3p to tumorigenesis is still lacking.

FBXW7 (F-box and WD40 domain protein 7) is a F-box WD40 protein and functions as a substrate recognition subunit of the SCF (SKP1/CUL1/F-box protein) E3 ubiquitin ligase complex.²² FBXW7 has been characterized as a general tumor suppressor. As an E3 ligase, FBXW7 exerts its tumor suppressor function mainly by targeting a network of oncoproteins (such as mTOR,²³ cyclinE²⁴ and Notch²⁵) for ubiquitination and proteasome degradation. FBXW7 deletion is often observed in multiple human cancers including breast cancer and loss of FBXW7 results in tumorigenesis.²⁶ Previous studies on FBXW7 have mainly focused on the degradation of its downstream targets. However, discovering the upstream target of FBXW7 in tumors is also important. Several recent studies have shown that FBXW7 is the target of miRNAs and functions in tumorigenesis. For example, Ding et al. reported that in colon cancer, the effect of FBXW7 on adriamycin sensitivity was regulated by miR-223.²⁷ In breast cancer, it has been shown that some miRNAs (such as miR-27a, miR-194, miR-32 and MiR-182) can regulate the occurrence and progression of breast cancer by regulating the expression of FBXW7.²⁸⁻³¹ Although some efforts have been made to study the regulation of FBXW7, a detailed understanding of the upstream of FBXW7 and the mechanisms that link FBXW7 deficiency to tumorigenesis is still lacking.

Here, we identified that high level of miR-223-3p is associated with poor prognosis in breast cancer. Moreover, miR-223-3p promoted migration and invasion of breast cancer cells both in vitro and in vivo. Mechanistic investigation discovered that FBXW7 is a novel target gene of miR-223-3p. miR-223-3p exhibited its oncogenic function via targeting

FBXW7 in breast cancer cells. Interference FBXW7 expression can restore anti-miR-223-3p induced inhibition of breast cancer cell migration and invasion. Taken together, our results provide evidence that the miR-223-3p/FBXW7 pathway exerts tumorigenic and metastatic effects in breast cancer. It provides novel mechanistic insights into the development and progression of breast cancer.

METHODS

Cell lines and cell culture

All cells were purchased from the American Type Culture Collection (ATCC) where they were characterized by DNA-fingerprinting and isozyme detection. All cells were maintained according to ATCC protocol and were grown at 37°C with 5% CO₂/95% air atmosphere.

Patients and specimens

Samples were collected from breast cancer patients who underwent curative resection. Informed consent was obtained from patients between 2018 and 2021 at The Second Hospital of Shandong University. The RNAs were isolated from 54 breast cancer and paracancerous tissues for qRT-PCR analysis of miR-223-3p and FBXW7 expression levels. Study protocols were approved by the Ethics Committee of The Second Hospital of Shandong University, and written informed consent was obtained from patients based on the Declaration of Helsinki.

Western blotting

Cell lysates were prepared using RIPA lysis buffer supplemented with protease inhibitor cocktail. Western blotting was performed as previously described.³²

RNA isolation and real-time RT-PCR

RNA was isolated using the Trizol reagent. miRcute miRNA isolation kit was used for isolation of small RNA. The reverse transcription reaction for miRcute miRNA was performed using the first strand cDNA synthesis kit and for mRNA was RevertAid First Strand cDNA synthesis kit. qRT-PCR was performed using the SYBR Green PCR Master Mix and the ABI PRISM 7900HT real-time PCR detection system.

Cell motility and invasion assay

Wound healing and transwell assays were used to assess cell motility. Invasion of cells was measured in matrigel-coated

transwell inserts containing polycarbonate filters with 8 μm pores. The detail of each assay has been described in our previous studies.³²

In vivo metastasis assay

Nude mice (6 weeks old) were purchased from Shanghai Slac Laboratory Animal Co. Ltd. and maintained in microisolator cages. For metastasis assays, 3×10^6 cells suspended with 0.1 ml PBS were injected into the tail veins of nude mice. After 40 days, all the mice were euthanized with chloral hydrate. The lung and liver were dissected and stained with hematoxylin and eosin. All experiments were undertaken in accordance with the guidelines of Shandong University and the current experiments were approved by the Institutional Animal Care and Use Committee of Shandong University.

Dual luciferase reporter assay

The wild-type and mutant FBXW7 3'-UTR were amplified by PCR and inserted into luciferase vector to generate wt-FBXW7 and Mun-FBXW7, respectively. A total of 1.0×10^5 cells were seeded in a 24-well plate. Cells were cotransfected with miR-223-3p mimics and wild-type or mutant of FBXW7 3'-UTR luciferase reporters. After being cultured for 48h, the luciferase activities of cells were measured using the dual luciferase reporter assay system following the manufacturer's recommendations.

Statistical analysis

SPSS statistical software was used for the statistical analysis. The results are presented as mean \pm SD. The association between miR-223-3p and FBXW7 in breast cancer tissue was assessed by the X^2 test. The expression differences between two different groups were analyzed with the

Student's *t*-test. *p*-values < 0.05 were considered statistically significant.

RESULTS

High miR-223-3p level is associated with poor prognosis in breast cancer

To investigate the role of miR-223-3p in breast cancer progression, we measured the mRNA levels of miR-223-3p in 54 paired breast cancer tissues and adjacent normal tissues using qRT-PCR. The mRNA levels of miR-223-3p were significantly upregulated in tumor tissues compared to adjacent normal tissues (Figure 1a). To further verify the results, we tested the expression of miR-223-3p in six breast cancer cell lines and one normal breast epithelial cell (MCF-10A). Consistent with the observation, miR-223-3p was overexpressed in breast cancer cell lines compared to normal epithelial cells (Figure 1b). Moreover, in analyzing the expression of miR-223-3p in 54 paired breast cancer and adjacent normal tissues, we found that miR-223-3p expression was positively correlated with the clinical stage of breast cancer (Figure 1c). Together, these results suggest that miR-223-3p is overexpressed in breast cancer. A high level of miR-223-3p expression could contribute to breast cancer progression and is a prognostic predictor of poor clinical outcome in breast cancer patients.

miR-223-3p promotes migration, invasion and epithelial-mesenchymal transition (EMT) of breast cancer cells

In order to test the oncogenic activity of miR-223-3p in breast cancer cells, MCF10A cells were used to establish miR-223-3p overexpressing cell line (designated as MCF10A-miR-223-3p) and MDA-MB-231 cells was used to establish miR-223-3p silencing cell line (designated as MDA-MB-231-Anti-miR-223-3p) by viral transduction. The levels of miR-223-3p in these resultant cell lines were

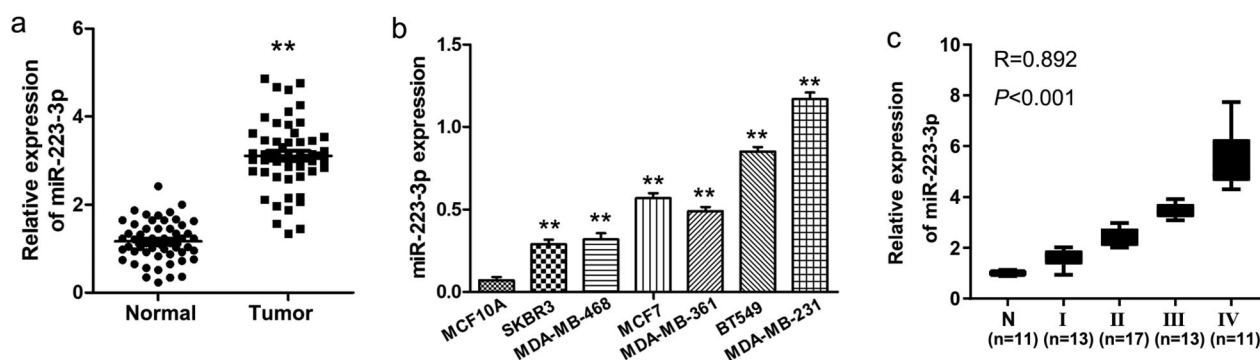


FIGURE 1 The analysis of miR-223-3p in breast cancer. (a) The miR-223-3p expression levels in 54 paired breast cancer tissues and corresponding normal tissues were measured by qRT-PCR. (b) The miR-223-3p expression levels in six breast cancer cell lines and normal breast epithelial cells (MCF-10A) were measured by qRT-PCR. (c) miR-223-3p expression correlated with clinical stage of breast cancer. $**p < 0.01$ is based on Student's *t*-test

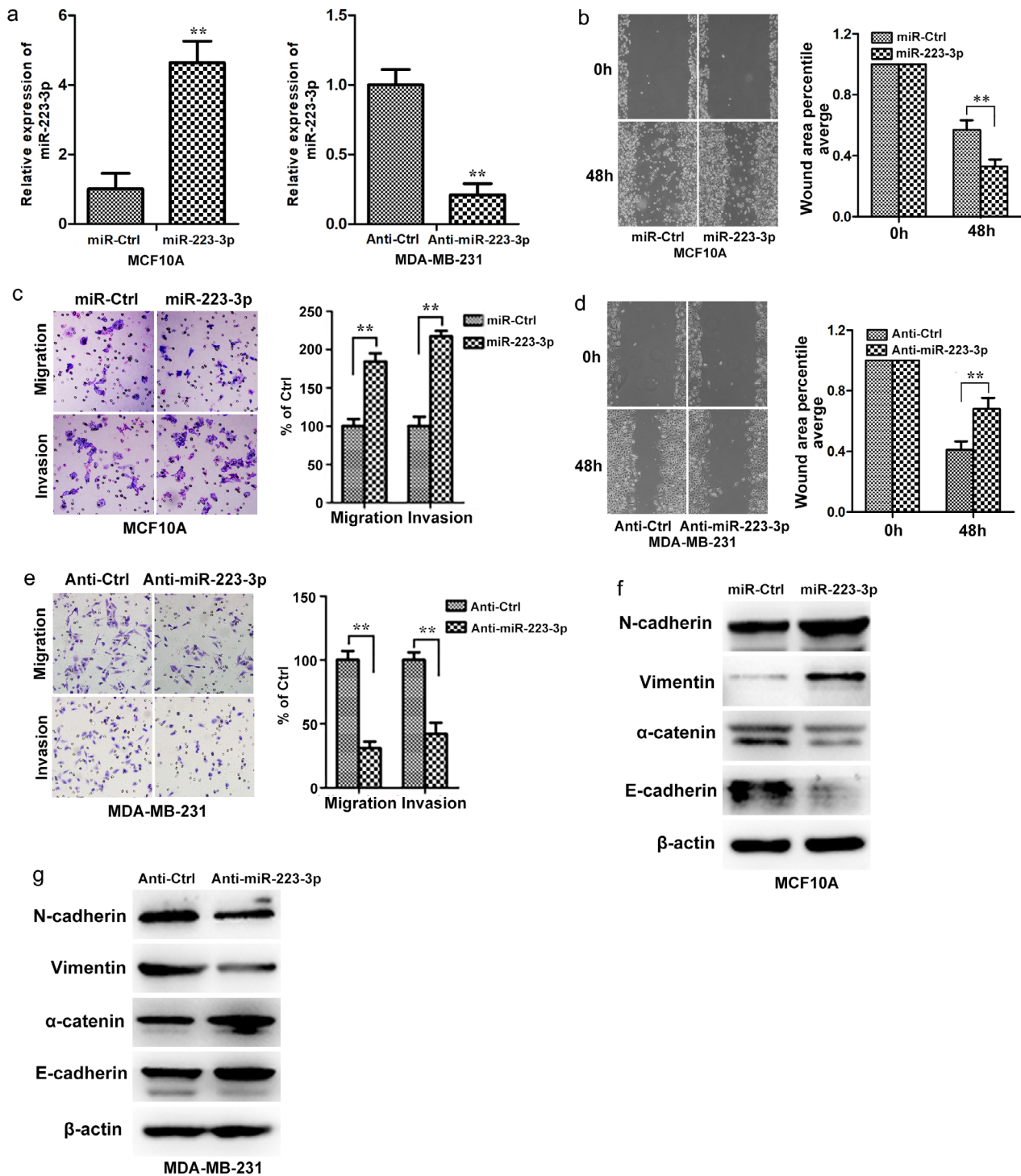


FIGURE 2 miR-223-3p promotes breast cancer cells migration, invasion and EMT in vitro. (a) The levels of miR-223-3p in overexpressing or silencing cell lines were verified by qRT-PCR. (b, c) The influence of miR-223-3p on migration and invasion abilities of MCF10A-miR-223-3p and control cells was determined by wound-healing (b), uncoated or Matrigel-coated transwell assay (c). The graphs show quantitative analysis. (d, e) The influence of miR-223-3p on migration and invasion abilities of MDA-MB-231-Anti-miR-223-3p and control cells was determined by wound-healing (d), uncoated or Matrigel-coated transwell assay (e). The graphs show quantitative analysis. (f, g) Expression of epithelial and mesenchymal markers were analyzed by western blotting in MCF10A-miR-223-3p cells (f) and MDA-MB-231-anti-miR-223-3p cells (g) or control cells

verified by qRT-PCR (Figure 2a). We used these cell lines to examine whether miR-223-3p could modulate the migratory and invasive capacities of breast cancer cells. Wound healing assay was firstly used to assess the effect of miR-223-3p on cell

migration. As shown in Figure 2b, overexpression of miR-223-3p had a faster closure of the wound area of MCF10A cell compared to its control cell. This result was further confirmed by Boyden chamber assay (Figure 2c, top panel). Moreover,

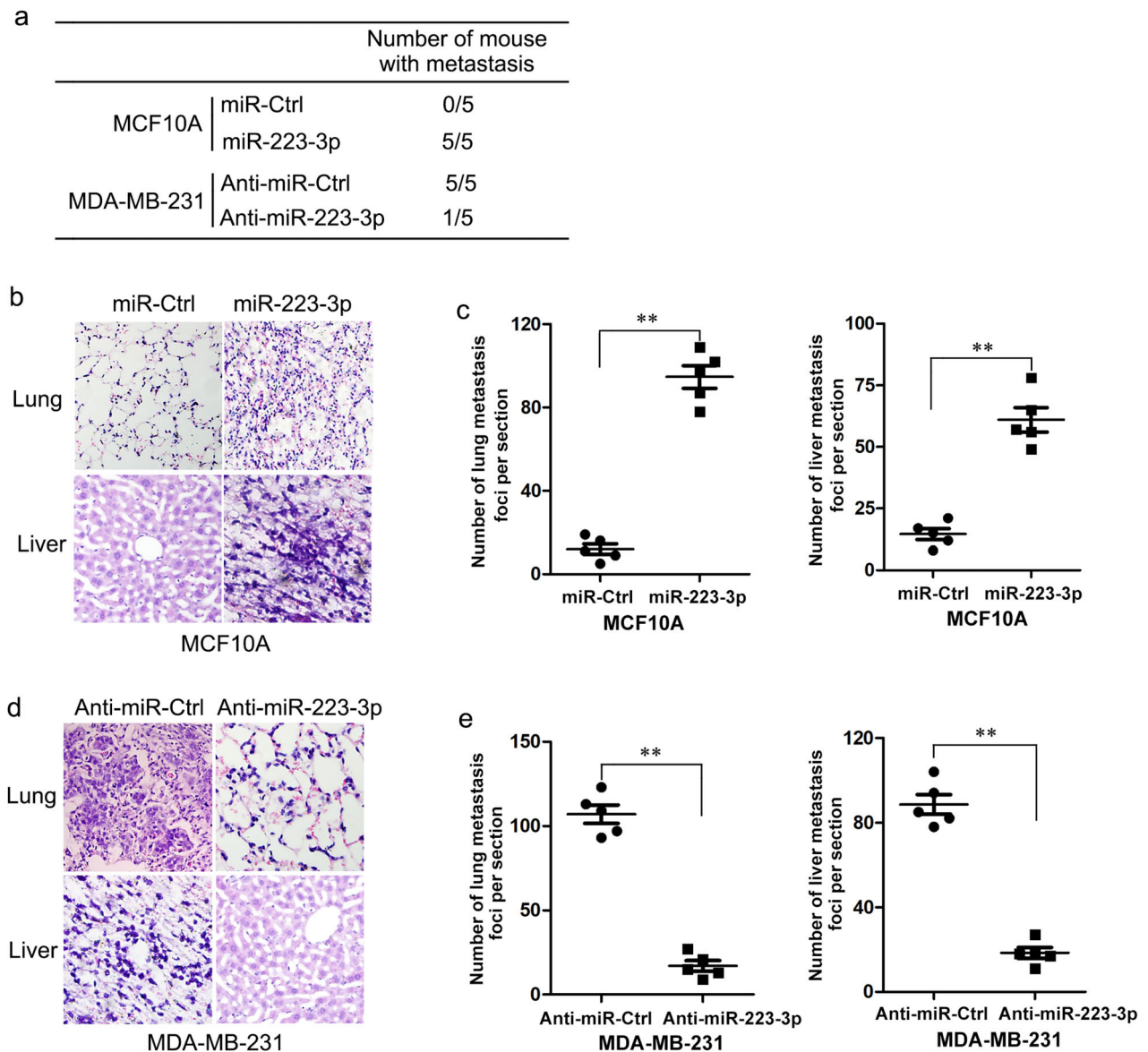


FIGURE 3 miR-223-3p promotes breast cancer cells metastasis in vivo. (a) Total numbers of nude mice with distant metastasis at 40 days after injection of MCF10A-miR-223-3p, MDA-MB-231-anti-miR-223-3p or control cells into tail vein. (b, c) Representative hematoxylin and eosin staining (b) and numbers of metastatic foci per section (c) in the lung and liver of individual mice injected with MCF10A-miR-223-3p or control cells. (d, e) Representative hematoxylin and eosin staining (d) and numbers of metastatic foci per section (e) in lung and liver of individual mouse with injection of MDA-MB-231-anti-miR-223-3p or control cells. $n = 5$ for tail-vein injection. $**p < 0.01$ is based on Student's t -test

overexpressed miR-223-3p cells showed a greater degree of invasion through Matrigel assay (Figure 2c, bottom panel). In contrast, decreased the expression of miR-223-3p dramatically inhibited the migration and invasion capacity of MDA-MB-231 cell (Figure 2d,e). These findings suggest that miR-223-3p promotes migratory and invasive potentials of breast cancer cells.

Recently, a growing number of studies reported that miRNAs play a pivotal role in regulating the EMT (epithelial-mesenchymal transition) program.^{5,33} It is well known that EMT is a major event that plays a crucial role in tumor progression and metastasis.³² Next, we detected the effect of miR-223-3p on EMT by examining the expression of EMT markers. We found that miR-223-3p overexpression increased the expression of mesenchymal markers (N-

cadherin and vimentin) and decreased the levels of epithelial markers (E-cadherin and α -catenin) in MCF10A cells (Figure 2f). Conversely, miR-223-3p silencing decreased the levels of mesenchymal markers, and increased the expression of epithelial markers (Figure 2g). Together, these results suggested that miR-223-3p is a regulator of EMT in breast cancer cells.

miR-223-3p promotes breast cancer cell metastasis in vivo

In order to investigate whether miR-223-3p was relevant for metastasis in vivo. MCF10A-miR-223-3p, MDA-MB-231-Anti-

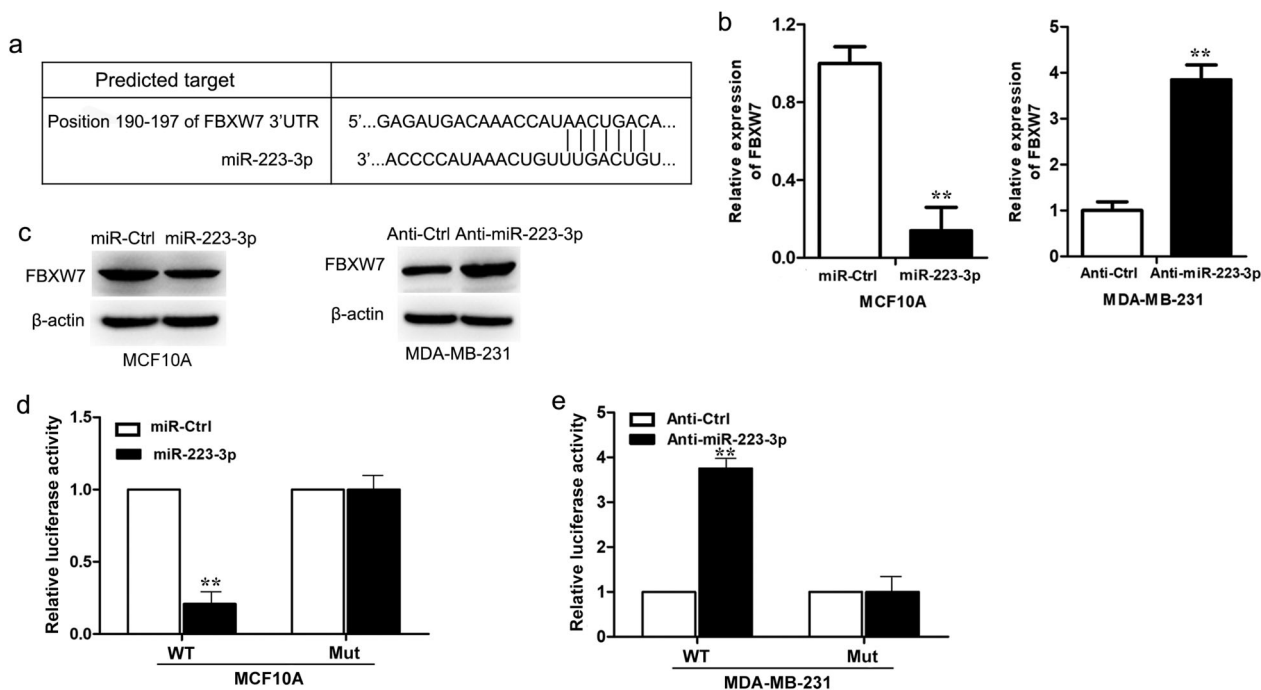


FIGURE 4 FBXW7 is a target gene of miR-223-3p. (a) Predicted binding of miR-223-3p to the 3'-UTR of FBXW7. (b, c) qRT-PCR and western blotting analysis of the effect of overexpression or silencing miR-223-3p on FBXW7 mRNA (b) and protein (c) levels in indicated breast cancer cells. (d) The luciferase activity was assayed in MCF10A-miR-223-3p and its control cells. (e) The luciferase activity was assayed in MDA-MB-231-anti-miR-223-3p and its control cells. ** $p < 0.01$ is based on Student's *t*-test

miR-223-3p and their corresponding control cells were injected into nude mice through the tail vein. miR-223-3p overexpression not only significantly increased the number of mice with distant metastasis (Figure 3a), but also increased the number of metastatic tumors in both lung and liver of each mouse (Figure 3b,c). On the contrary, silencing miR-223-3p expression inhibited the metastatic behavior, both in terms of the number of mice with distant metastasis (Figure 3a) and the number of metastatic tumors in the lung and liver of each mouse (Figure 3d,e).

FBXW7 is a target gene of miR-223-3p

To investigate the molecular mechanism of miR-223-3p regulating the invasion and metastasis of breast cancer cells, we screened substrates recognized by miR-223-3p. We used target gene prediction websites (such as Targetscan and miRDB) to identify the target genes of miR-223-3p. The results from these websites all predicted FBXW7 contains a putative target for miR-223-3p in its 3'-UTR (Figure 4a). Thus, the expression of FBXW7 was examined by Western blotting and qRT-PCR in MCF10A and MDA-MB-231 cells. Consistent with our prediction, the expression of FBXW7 was significantly higher in miR-223-3p silenced cells compared with its control cells at both mRNA and protein levels. On the contrary, in miR-223-3p overexpressed cells, the mRNA and protein level of FBXW7 was decreased (Figure 4b,c). To determine whether miR-223-3p directly targets the FBXW7 gene, we cloned the 3'-UTR of the putative FBXW7 site into a luciferase construct.

Reporter assays revealed that overexpression miR-223-3p reduced luciferase activity (Figure 4d), and anti-miR-223-3p increased luciferase activity (Figure 4e). Mutation of miR-223-3p binding sites in the FBXW7 3'-UTR abrogated the miR-223-3p effect. Taken together, these results suggest that miR-223-3p targets the 3'-UTR of FBXW7 and downregulates FBXW7 expression.

FBXW7 is a mediator for miR-223-3p induced migration and invasion of breast cancer cells

To test whether miR-223-3p induced migration and invasion was mediated by FBXW7, shRNA was used to silence FBXW7 expression by virally transfecting MDA-MB-231-anti-miR-223-3p cells. Knockdown of FBXW7 expression in MDA-MB-231-anti-miR-223-3p cells resulted in an increase in migration and invasion capacities (Figure 5a). To investigate if FBXW7 mediates miR-223-3p induced metastasis in vivo, MDA-MB-231-anti-miR-223-3p cells with or without silencing FBXW7 were injected into nude mice through the tail vein. Silencing FBXW7 not only significantly increased the number of mice with distant metastasis (Figure 5b), but also dramatically increased the number of metastatic tumors in both lung and liver of each mouse (Figure 5c). These results demonstrated the critical role of FBXW7 in mediating miR-223-3p promoted metastasis behavior in breast cancer cells.

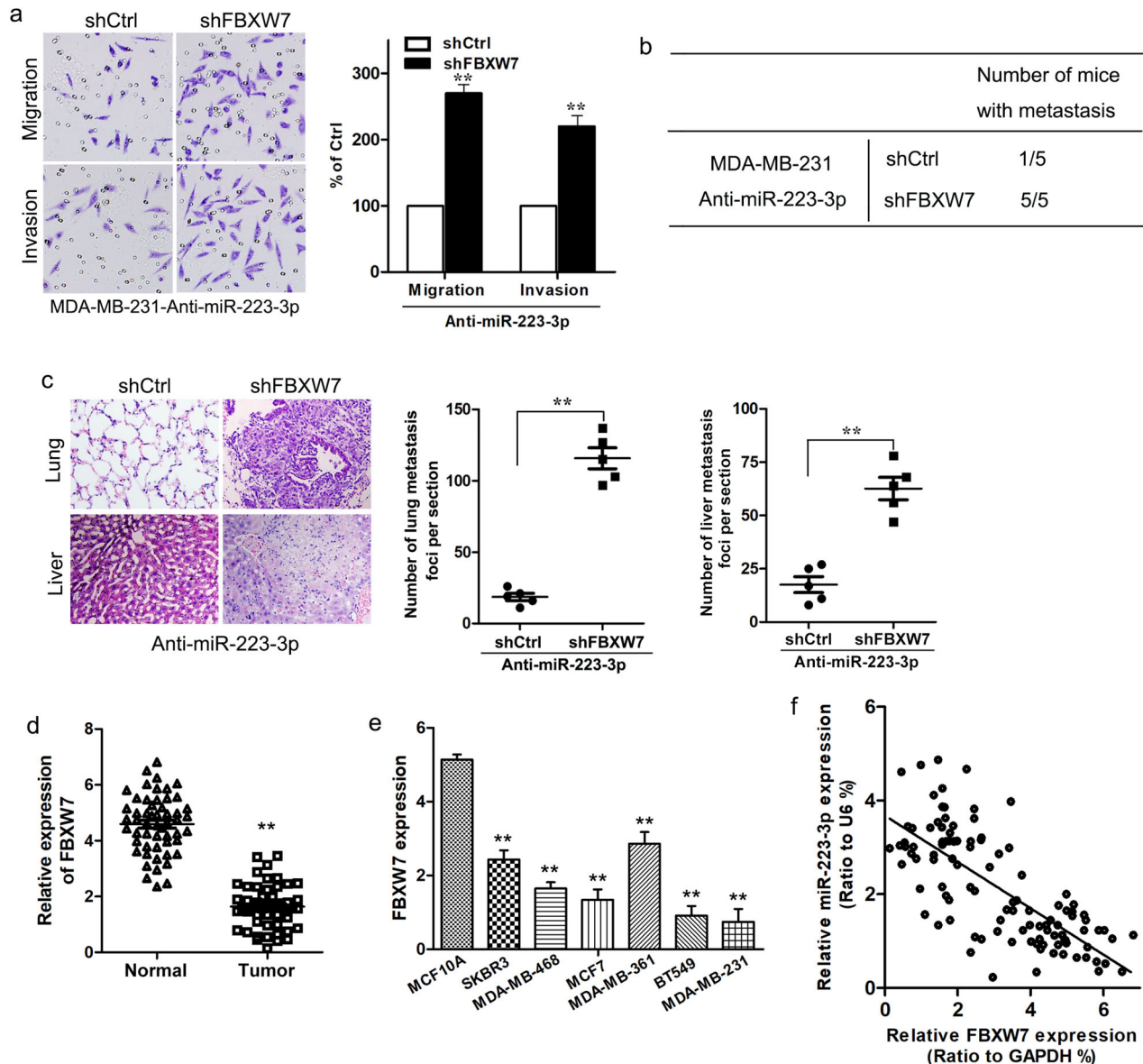


FIGURE 5 Interference of FBXW7 restored miR-223-3p mediated breast cancer cell migration, invasion and metastasis. (a) Cell migration and invasion was examined by uncoated or Matrigel-coated transwell assay after knockdown of FBXW7 expression in MDA-MB-231-anti-miR-223-3p cells. (b) Tumor metastasis was assayed after knockdown of FBXW7 expression in MDA-MB-231-anti-miR-223-3p cells. (c) Representative hematoxylin and eosin staining and numbers of metastatic foci per section in lung and liver of individual mouse after knockdown of FBXW7 expression in MDA-MB-231-anti-miR-223-3p cells. (d) The FBXW7 expression levels in 54 paired breast cancer tissues and corresponding normal tissues were measured by qRT-PCR. (e) The FBXW7 expression levels in six breast cancer cell lines and normal breast epithelial cells (MCF-10A) were measured by qRT-PCR. (f) A significant negative correlation between the levels of miR-223-3p and FBXW7 expression was observed in breast cancer specimens. $**p < 0.01$ is based on Student's *t*-test

To recognize the clinical correlation of FBXW7 and miR-223-3p, FBXW7 expression in the same human breast cancer tissues was analyzed. As expected, the mRNA level of FBXW7 was significantly downregulated in tumor tissues compared to adjacent normal tissues (Figure 5d). Similarly, FBXW7 expression was decreased in breast cancer cells compared to normal epithelial cells (Figure 5e). More importantly, statistical analysis showed that miR-223-3p expression was negatively correlated with FBXW7 level in breast cancer tissues (Figure 5f).

These results are consistent with our above analyses both in vitro and in vivo.

DISCUSSION

Aberrant expression of miRNAs has previously been identified to be involved in breast cancer development and progression.^{34–36} In this study, we found that the expression of miR-223-3p was significantly upregulated in breast cancer

tissues compared with adjacent normal tissues. miR-223-3p was also overexpressed in breast cancer cell lines compared with normal cell lines. Consistent with this observation, a high level of miR-223-3p was associated with poor prognosis in breast cancer patients. In our effort to elucidate the oncogenic activity of miR-223-3p, we found that overexpressed miR-223-3p promoted the migration and invasion of breast cancer cells *in vivo* and *in vitro*. Knockdown miR-223-3p expression inhibited breast cancer cell migration and invasion. These results imply that miR-223-3p may play an oncogenic role in breast cancer.

EMT is characterized by the loss of epithelial characteristics and acquisition of a mesenchymal phenotype which promotes tumor progression through conferring cells migratory and invasive properties.³² In this study, we found that breast cancer cells with overexpressed miR-223-3p could promote the EMT process, whereas silencing of miR-223-3p resulted in the reversal of EMT. Consistent with this observation, breast cancer cells with ectopic expression of miR-223-3p contributed to increase the numbers of distant metastasis *in vivo*. These findings provide an explanation for the observation that breast cancer patients with high levels of miR-223-3p in tissue samples have a significantly shorter overall survival.

Mechanistically, accumulating studies suggest miRNAs exert their function mainly by regulating its target genes and interacting with multiple pathway networks. Therefore, to elucidate the mechanism how miR-223-3p modulates migration and invasion in breast cancer cells, we screened the candidate targets of miR-223-3p using target gene prediction websites. Among these predicted targets, FBXW7 was selected for analyses as FBXW7 was reported to function as a general tumor suppressor to regulate a network of proteins with central roles in cell cycle, growth and division.^{37,38} Recent studies have reported that FBXW7 is a target of miR-223-3p and functions in the regulation of cancers. For instance, Zhang et al. reported that RASA1 functions in renal cell carcinoma by decreasing miR-223-3p expression and enhancing the expression of FBXW7.³⁹ Hou et al. demonstrated circ_0000094 impedes T cell acute lymphoblastic leukemia progression by modulating the miR-223-3p/FBXW7 axis.⁴⁰ Additionally, miR-223-3p regulates cell growth and apoptosis of testicular germ cell tumors by suppressing FBXW7 expression.⁴¹ However, although the regulation of miR-223-3p to FBXW7 has been reported, the potential role of miR-223-3p/FBXW7 axis in breast cancer progression remains unclear. In the present study, qRT-PCR and western blotting assay showed overexpression of miR-223-3p could decrease the expression of FBXW7 in breast cancer cells, whereas knockdown of miR-223-3p could elevate FBXW7 expression. In addition, luciferase activity reporter assay indicated miR-223-3p could directly bind with 3'-UTR of FBXW7. Thus, we concluded that miR-223-3p plays an oncogenic role by directing target FBXW7, and consequently promotes cell EMT and invasion *in vitro* and metastasis *in vivo* in breast cancer.

In conclusion, our study uncovered a physical and functional relationship between miR-223-3p and FBXW7. Our results demonstrated that the increased miR-223-3p induced EMT, migration and invasion of breast cancer cells is partly through regulating FBXW7 expression. We discovered a novel miR-223-3p/FBXW7 signaling pathway which plays an important functional role in breast cancer progression and metastasis. Our findings provide a novel therapeutic avenue for improving current anticancer therapies by manipulating miR-223-3p levels in clinical practice.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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