53BP1 suppresses epithelial–mesenchymal transition by downregulating ZEB1 through microRNA-200b/429 in breast cancer

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Breast cancer is the second most common cause of cancer-related deaths among women in the USA. Approximately 230,000 women were diagnosed with, and 40,000 died from, invasive breast cancer in the USA in 2014.¹,² Metastasis is the main reason for most breast cancer-related deaths. Cancer cells migrate from the primary tumor and invade and re-establish at distant sites.³ Although conventional chemotherapies and radiotherapies are used, their effects are minimal for metastatic breast cancer, thus it is generally incurable.⁴ This treatment failure is due, in part, to the mechanisms of metastasis of breast cancer not being clarified. Once the key mechanism of metastasis is uncovered, corresponding therapies can be researched and developed.

Epithelial–mesenchymal transition (EMT) is a process in which epithelial cells lose their polarity and acquire the properties of mesenchymal cells.⁵,⁶ In recent years, EMT has been the research hotspot in cancer-related research because of its correlations with many important steps in cancer progression, including stemness,⁷ drug resistance,⁷ and microenvironmental regulation.⁸ Epithelial–mesenchymal transition is known to be a central mechanism for the metastasis and invasiveness of breast cancer. The function of EMT in enhancing migration and invasion of cancers has drawn great attention from scientists. During the regulation of EMT, many oncogene and tumor suppressor genes play crucial roles.⁹–¹² The new tumor suppressor gene p53 binding protein 1 (53BP1) has been the research focus of our team over recent years. It is mainly reported as an important regulator of the cellular response to DNA double-strand breaks.¹³,¹⁴ Our team first proposed that 53BP1 might function as a tumor suppressor gene in breast cancer. However, it remains unknown whether 53BP1 can regulate EMT in human cancers, including breast cancer.

In this study, we found that 53BP1 negatively regulated the EMT of breast cancer through microRNA (miR)-200b/429-mediated ZEB1 downregulation. Our results supported that 53BP1 suppressed tumor function by negatively regulating EMT and might be a crucial regulator of breast cancer migration and invasion.

Materials and Methods

Cell culture and reagents. Human breast cancer cell lines, MDA-MB-231 and MCF-7, were obtained from ATCC (Rockville, MD, USA) and cultured in DMEM (Gibco, Rockville, IN, USA) containing 10% FBS (Clark Bioscience, Seabrook,
Rabbit anti-fibronectin antibody was from Abcam (Cambridge, UK). Rabbit anti-ZEB1 antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Other antibodies were all from Cell Signaling Technology (Beverly, MA, USA). MicroRNA mimics and inhibitors were obtained from Guangzhou RiboBio (Guangzhou, China). Small interfering RNAs of ZEB1 were from Sigma-Aldrich (St. Louis, MO, USA).

Plasmid construction and transfection. The plasmid construction was carried out as described previously.\(^{15}\) For RNA interference of 53BP1, the target sequences of sense shRNA were: GCCAGGGUUCAGAGGAAG. The pSuper-Neo-GFP from OligoEngine (Seattle, WA, USA) vector was used. For overexpression of 53BP1, the Addgene (Cambridge, MA, USA) plasmid 19836: N-Myc-53BP1 WT pLPC-Puro was used.\(^ {16}\) Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to transfect the plasmids according to the manufacturer’s protocol.

Quantitative RT-PCR analysis. RNA was extracted by using TRIzol (Takara, Dalian, China) reagents. Total RNA was used for RT reactions and quantitative (q)RT-PCR according to the manufacturer’s protocol (Takara). The experiments were repeated in triplicate, at minimum.

Western blot analysis. Cells were lysed with radio immunoprecipitation assay buffer (Millipore, Bedford, MA, USA). Equal amounts of protein were separated on a SDS gel and electrotransferred to PVDF membranes (Millipore, Bedford, MA, USA). Signals were detected using a Luminescent Image analyzer (GE Healthcare Bio-Sciences, Uppsala, Sweden). β-Actin was used as the control.

Immunofluorescence staining. Cells were grown on coverslips in 24-well plates. After washing in PBS, cells were fixed with 4% paraformaldehyde. Cells were blocked with 10% normal goat serum in PBS, followed by rabbit primary antibodies and rhodamine-conjugated anti-rabbit secondary antibody (Jackson Immuno Research, West Grove, PA, USA). The membranes were fixed and stained using antifading medium (Beyotime Institute of Biotechnology, Jiangsu, China). The fluorescence signal was examined with a fluorescence microscope (Olympus, Tokyo, Japan).

Transwell migration and invasion assay. For the migration assay, 1 × 10⁵ cells were plated in the upper chamber and allowed to migrate to the lower chamber (BD Biosciences, San Jose, CA, USA). After specified times, the Transwell membranes were fixed and stained using crystal violet. Cells were counted under a light microscope (Olympus) for at least 10 random visual fields. The invasion assay was similar to the migration assay, except that the upper side of the membranes was coated with Matrigel (BD Biosciences).

Immunohistochemistry. The sections from tumors in 4–5-week-old female SCID mice previously injected with control or 53BP1 knockdown MCF-7 cells and overexpressed MDA-MB-231 cells\(^ {17}\) were used for detection of the expression of EMT-related markers. The streptavidin–peroxidase–biotin reagent kit was from Zhongsan Biotechnology (Beijing, China). Immunohistochemistry was carried out according to the manufacturer’s protocol. Tissue sections were then incubated with streptavidin–HRP complex and followed by hematoxylin. For negative control, the antibody solution was replaced with PBS.

Patients and tissue samples. A total of 18 fresh specimens of breast cancer tissues were collected between 2009 and 2011. For the research use of these clinical materials, prior patient consent and approval from the institutional research ethics committee were obtained. All the diagnoses were made by two pathologists according to the guidelines of the Pathology and Genetics of Tumours of the Breast and Female Genital Organs of the World Health Organization Classification of Tumours.

Statistical analysis. The results were analyzed using srs 18.0 software (SPSS, Chicago, IL, USA). Each experiment was carried out at least three times. The data were expressed as mean ± SEM. Two-tailed Student’s t-test was used to calculate the statistical significance. Bivariate correlations between study variables in tissues were calculated by Pearson’s rank correlation coefficients. P-values <0.05 were considered statistically significant.

Results

53BP1 suppressed EMT and migration in breast cancer cells. In order to investigate the functions of 53BP1, we previously established 53BP1 overexpressed MDA-MB-231 cell lines (MDA-MB-231-53BP1 cells) and 53BP1 knockdown MCF-7 cell lines (MCF-7-sh53BP1 cells).\(^ {17}\) During the culture of these cells, we observed morphology changes. MDA-MB-231-53BP1 cells had an epithelial-like morphology. Consistent with this observation, MCF-7-sh53BP1 cells showed a mesenchymal-like morphology, including a greater number of spindles (Fig. 1a). We next examined the expressions of EMT markers by using Western blot and qRT-PCR methods. The results showed that, in MDA-MB-231-53BP1 cells, the expression of epithelial marker E-cadherin was increased, whereas the expression of mesenchymal markers ZEB1, vimentin, and fibronectin was obviously reduced. MCF-7-sh53BP1 cells showed downregulation of the expression of epithelial marker and upregulation of the expression of mesenchymal markers (Fig. 1b). The expressions of other EMT transcription factors (TFs) were slightly regulated but less obvious than ZEB1 (Fig. 1b, right). The expressions of epithelial marker E-cadherin and mesenchymal markers including ZEB1 and fibronectin were also confirmed by immunofluorescence staining, as shown in Figure 1(c).

We next examined whether 53BP1 affected the migration and invasion of breast cancer cells by Transwell assay. We found that 53BP1 could suppress the migration and invasion in MDA-MB-231-53BP1 cells, whereas this activity was increased in MCF-7-sh53BP1 cells (Fig. 2).

Together, these data suggested that 53BP1 suppressed the EMT and tumor migration and invasion in breast cancer cells.

53BP1 suppressed EMT and migration and invasion by targeting ZEB1 through upregulating miR-200b/429 in breast cancer cells. We found that ZEB1 was regulated most significantly in the expression of EMT TFs that suppressed E-cadherin (Fig. 1b). Thus, we speculated that 53BP1 mainly regulated EMT through targeting ZEB1. A review of published works revealed that the miR-200 family, including miR-200a/b/c, miR-141, and miR-429, were reported to directly target the E-cadherin transcriptional repressors ZEB1. Therefore, we detected the expression of the miR-200 family in MDA-MB-231-53BP1 cells and MCF-7-sh53BP1 cells compared with control cells. Using qRT-PCR, we confirmed that miR-200b and miR-429 were obviously upregulated in MDA-MB-231-53BP1 cells and decreased in MCF-7-sh53BP1 cells (Fig. 3a). We also validated the expressions of other miRNAs that have potential binding sites for ZEB1 predicted by TargetScan, PicTar, Miranda, and miRDB, including miR-23b, miR-199a, miR-96, and miR-150. Results showed that some of these miRNAs were
53BP1 suppressed EMT in breast cancer

(a) MDA-MB-231

Con  53BP1

shCon  sh53BP1

(b) MDA-MB-231

Con  53BP1

53BP1  53BP1

E-cadherin  E-cadherin

ZEB1  ZEB1

Vimentin  Vimentin

Fibronectin  Fibronectin

β-actin  β-actin

MCF-7

Con  53BP1

shCon  sh53BP1

53BP1  53BP1

E-cadherin  E-cadherin

ZEB1  ZEB1

Vimentin  Vimentin

Fibronectin  Fibronectin

β-actin  β-actin

(c) MDA-MB-231

Con  53BP1

shCon  sh53BP1

E-cadherin

ZEB1

Fibronectin
downregulated by 53BP1 knockdown and upregulated by 53BP1 overexpression. However, their expression changes by 53BP1 were not as significant as miR-200b and miR-429.

In order to examine the functions of miR-200b⁄429, we transfected MDA-MB-231-53BP1 cells and MCF-7-sh53BP1 cells with miR-200b/429 inhibitors and mimics, respectively. Using Western blot analysis, we confirmed changes in ZEB1, E-cadherin, vimentin, and fibronectin expressions after treatment with miR-200b/429 inhibitors or mimics in 53BP1 overexpressed and knockdown cells, respectively (Fig. 3b,c). We also transfected siRNAs of ZEB1 in MCF-7-sh53BP1 cells to observe the regulation of miR-200b and miR-429 (Fig. 3d). It showed that after downregulating ZEB1, the expressions of miR-200b and miR-429 were not obviously regulated, which indicated that 53BP1 indeed regulated ZEB1 through miR-200b⁄429.

Furthermore, treatments with miR-200b/429 inhibitors and mimics reversed the effects of 53BP1 on migration and invasion in MDA-MB-231-53BP1 cells and MCF-7-sh53BP1 cells (Fig. 4), indicating that miR-200b/429 mediated 53BP1’s negative regulation of migration and invasion.

Together, these data suggested that loss of 53BP1 inhibited miR-200b and miR-429 expression, thus targeting the expression of ZEB1 and enhancing EMT and breast cancer cell migration and invasion.

53BP1 suppressed EMT in vivo in breast cancer. Our results were confirmed in vivo in a tumor xenograft mice model conducted previously. Immunohistochemistry analysis of MDA-MB-231-53BP1 tumors showed that ZEB1 and vimentin expression decreased, whereas E-cadherin expression increased. Consistently, ZEB1 and vimentin increased and E-cadherin expression decreased in MCF-7-sh53BP1 tumor sections (Fig. 5). Taken together, these data indicated that 53BP1 is a positive regulator of E-cadherin through downregulating the expression of the E-cadherin repressor ZEB1.

53BP1 positively correlated with miR-200b⁄429 and negatively correlated with ZEB1 in clinical samples and could function as a tumor suppressor in clinical breast cancer. The expression levels of 53BP1, miR-200b/429, and ZEB1 were detected in 18 breast cancer tissues. As indicated in Figure 6(a,b), there existed a positive correlation between the expression levels of 53BP1 and miR-200b (r = 0.4980, P = 0.0355) and miR-429 (r = 0.5175, P = 0.0278). The tissues with the relatively high 53BP1 expression levels showed high levels of miR-200b and miR-429 as determined by qRT-PCR. It was also found that 53BP1 was negatively correlated with ZEB1 expression (r = -0.4980, P = 0.0355) (Fig. 6c).

Furthermore, our analysis revealed that downregulation of 53BP1 was significantly associated with lymph node metastasis.
Discussion

Metastasis is a key step in the progression of breast cancer. Most breast cancer-related deaths are due to metastasis. For metastatic breast cancer, the effects of conventional treatments are limited. One of the reasons is that the mechanisms of breast cancer metastasis are still not fully known. Breast cancer is a heterogeneous disease associated with variations in gene expression.\(^{(4,18)}\) Overexpression of oncogenes and loss of tumor suppressor genes play important roles in the progression of breast cancer. The finding of new genes that are crucial in metastasis is helpful for future treatment of metastatic breast cancer.

\(53BP1\) was first proposed as a tumor suppressor gene in breast cancer by our team. Previously, we found that \(53BP1\) loss could rescue BRCA1 deficiency and was associated with triple-negative and BRCA-mutated breast cancers.\(^{(19)}\) Low \(53BP1\) expression was reported to be associated with increased local recurrence in breast cancer patients treated with breast-conserving surgery and radiotherapy.\(^{(20)}\) In 2012, we formally presented that \(53BP1\) functions as a tumor suppressor gene in breast cancer.\(^{(17)}\) \(53BP1\) could significantly inhibit cell proliferation and the invasiveness and metastasis in breast cancer both in vitro and in vivo by the inhibition of nuclear factor-\(\kappa\)B through \(miR-146a.\)\(^{(17)}\) However, the mechanism of metastasis in breast cancer is complex. Epithelial–mesenchymal transition is a key step in the regulation of cancer metastasis. The aim of our study was to research the tumor suppressor gene role of \(53BP1\) from a new angle.

In this study, we investigated a novel role for \(53BP1\) in breast cancer. Consistent with our previous reports, this study indicated that \(53BP1\) could modulate EMT, which is crucial for tumor migration and invasion. Loss of E-cadherin is the hallmark of EMT. Several TFs that directly regulate the expression of E-cadherin play crucial roles in development of EMT, including Snail, Slug, and \(ZEB1.\) In this study, we found that overexpression of \(53BP1\) led to increase of E-cadherin. After expression detection of transcriptional suppressors, \(ZEB1\) was found to decrease most significantly. Therefore, we speculated that \(53BP1\) suppressed EMT of breast cancer mainly through targeting \(ZEB1.\) In the regulation of \(ZEB1,\) miRNAs play crucial roles.

MicroRNAs are small non-coding RNA that can interact with miRNAs and regulate their expression. One miRNA can regulate several specific miRNAs and one miRNA can be regulated by several miRNAs. MicroRNAs have been reported to play crucial roles in many cancers. In breast cancer, miRNAs...
function in several physiological processes, including development, proliferation, migration and invasion, differentiation, and apoptosis. Through a review published works and prediction by TargetScan, PicTar, Miranda, and miRDB, several miRNAs were screened out to target ZEB1, including the miR-200 family (miR-200a/b/c, miR-141, and miR-429), miR-23b, miR-199a, miR-96, and miR-205. By using qRT-PCR, we detected the expressions of some miRNAs in MDA-MB-231-53BP1 cells and MCF-7-sh53BP1 cells compared with control cells. We found that the expressions of miR-200b and miR-429 were regulated most significantly. Except for miR-200b and -429, miR-200a in MDA-MB-231-53BP1 cells was slightly up-regulated and miR-200c in MCF-7-sh53BP1 cells was down-regulated. The miR-200 family consists of the miR-200b/200a/429 cluster on chromosome 1 and the miR-200c/141 cluster on chromosome 12. Transcription factors together with epigenetic modulation demonstrated the complex network controlling the miR-200 family. The miR-200b/200a/429 and miR-200c/141 clusters were regulated by diverse mechanisms. For example, both miR-200 clusters were associated with local CpG enrichments but had different methylation patterns. Therefore, 53BP1 might regulate two miR-200 clusters through different ways in different cells, but mainly promote miR-200b/429 in these two breast cancer cell lines.

In order to confirm the roles of these two miRNAs in 53BP1 regulating EMT, cells were treated with miR-200b/429 mimics and inhibitors. The results showed that suppression roles of 53BP1 on EMT and invasion of breast cancer were clearly reversed. In addition, it was reported that the miR-200 family could regulate the EMT by inhibiting ZEB1. In contrast, ZEB1 repressed the expression of the miR-200 family, suggesting that both ZEB1 and miR-200 could share a double-negative feedback loop. Therefore, we transfected the siRNAs of ZEB1 in MCF-7-sh53BP1 cells to investigate this mechanism. The results showed that, after regulating the expression of ZEB1, miR-200b and -429 did not change significantly, which indicated that the double-negative feedback loop did not work and 53BP1 indeed regulated ZEB1 through miR-200b and -429.

The miR-200 family was reported as a WT p53 target that regulated p53-mediated EMT suppression by targeting ZEB1. It was reported that p53 mutants had the opposite

![Fig. 4](https://www.wileyonlinelibrary.com/journal/cas)

**Fig. 4.** Negative effects of 53BP1 on migration and invasion were mediated by miR-200b and miR-429 in breast cancer cells. (a) MDA-MB-231-53BP1 overexpressed cells were transfected with miR-Control (Con) and miR-200b/429 inhibitors (Inh). Migration and invasion assays were carried out. (b) MCF-7-sh53BP1 cells were transfected with miR-Control and miR-200b/429 mimics. Migration and invasion assays were carried out. All experiments were performed at least three times. Error bars, ±SEM. *P < 0.05 versus miR-Control, #P < 0.05 versus miR-Control (Student’s t-test).
effects of WT p53, which led to decreased miR-200 expression.\(^\text{(31)}\) In our results, 53BP1 regulated miR-200b and -429 both in mutant p53 MDA-MB-231 cells and WT p53 MCF-7 cells. Therefore, we speculated that 53BP1 did not work in a p53-dependent manner. The mechanisms of miR-200b and -429 regulation of 53BP1 need to be investigated more in our future studies.

To further investigate whether 53BP1 could suppress EMT by ZEB1 expression, we analyzed the expressions of epithelial marker E-cadherin and mesenchymal markers ZEB1 and vimentin in vivo in a tumor xenograft mice model.\(^\text{(17)}\) The results of immunohistochemistry showed that ZEB1 and vimentin expression decreased, whereas E-cadherin expression increased when 53BP1 was overexpressed, indicating that 53BP1 is a positive regulator of E-cadherin through downregulating the expression of the E-cadherin repressor ZEB1. Our previous study indicated that 53BP1 could inhibit lung metastasis in vivo. After being injected into the lateral tail veins of nude mice, mice with MDA-MB-231 overexpressing 53BP1 developed less metastatic foci than the control mice.\(^\text{(17)}\) These results verified the tumor suppressor gene effect of 53BP1 in vivo.

Studies with clinical tissue samples could provide more convincing evidence. In this study, we used 18 breast cancer tissues collected between 2009 and 2011 to extract the total RNA and detect the expressions of 53BP1, miR-200b/-429, and ZEB1 by using qRT-PCR. As indicated in Figure 6, expression of 53BP1 was significantly positively correlated with miR-200b and miR-429 and negatively correlated with ZEB1 in clinical samples and could function as a tumor suppressor in clinical breast cancer. The relative expression levels of miR-200b (a), miR-429 (b), and ZEB1 (c) were plotted against that of 53BP1 in each sample. Bivariate correlations between study variables calculated by Spearman’s rank correlation coefficients. (d) Expression of 53BP1 in primary breast cancer with or without lymph node metastasis. Student’s t-test was carried out.

Fig. 5. 53BP1 regulated ZEB1 expression and suppressed epithelial-mesenchymal transition in vivo. Immunohistochemistry of 53BP1 and epithelial-mesenchymal transition markers, including E-cadherin, ZEB1, and vimentin, from sections of tumor xenograft mice injected with MDA-MB-231-Control (Con) cells, MDA-MB-231-53BP1 overexpressed cells, MCF-7-shControl (shCon) cells, and MCF-7-sh53BP1 cells. All experiments were carried out at least three times.

Fig. 6. 53BP1 was positively correlated with miR-200b and miR-429 and negatively correlated with ZEB1 in clinical samples and could function as a tumor suppressor in clinical breast cancer. The relative expression levels of miR-200b (a), miR-429 (b), and ZEB1 (c) were plotted against that of 53BP1 in each sample. Bivariate correlations between study variables calculated by Spearman’s rank correlation coefficients. (d) Expression of 53BP1 in primary breast cancer with or without lymph node metastasis. Student’s t-test was carried out.

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200b \((r = 0.4691, P = 0.0495)\) and miR-429 \((r = 0.5175, P = 0.0278)\). It was also negatively correlated with the expression of \(ZEB1\) \(= P_{0.4980, P = 0.0355}\). These results showed the correlation between \(53BP1\) and miR-200b/429 and \(ZEB1\) at the clinical level. Experiments \(in vitro\) and \(in vivo\) both indicated that \(53BP1\) suppressed the migration and invasion of breast cancer cells. Therefore, we calculated the relationship between \(53BP1\) and lymph node metastasis. It was shown that the expression of \(53BP1\) in patients without lymph node metastasis was significantly higher than those who had positive lymph node \((P < 0.001)\). These results prompted the clinical application value of \(53BP1\). Because of the lack of follow-up information of these 18 breast cancer patients, we did not investigate the correlation between \(53BP1\) and distant metastasis. In future research, this could be further discussed.

In conclusion, our findings provided evidence for the tumor suppressive roles of \(53BP1\) in EMT and metastasis in breast cancer \(in vitro, in vivo\), and at the clinical level. We propose that \(53BP1\) decreased \(ZEB1\) expression through the upregulation of miR-200b and miR-429, thus inhibiting EMT and migration and invasion. Therefore, loss of \(53BP1\) might be a critical step in metastasis in breast cancer. In the future, \(53BP1\) could be a new potential drug target for treatment of metastatic breast cancer.

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Disclosure Statement

The authors have no conflict of interest.

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