53BP1 functions as a tumor suppressor in breast cancer via the inhibition of NF-κB through miR-146a

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p53-binding protein-1 (53BP1) plays a critical role in cell cycle checkpoint and DNA repair activities. Recently, 53BP1 was recognized as a potential tumor suppressor gene. In this study, we investigated its tumor suppressor function in breast cancer. In clinical samples, we observed a lower level of 53BP1 expression in the cancer lesions than in the matched non-tumor tissues. Furthermore, the 53BP1 level showed a gradual decrease during the progression from precancerous to cancer lesion. Ectopic expression of 53BP1 can significantly inhibit cell proliferation and curb the invasiveness in breast cancer cell lines, whereas knockdown of 53BP1 by RNA interference had the opposite effects. Additionally, 53BP1 markedly inhibited xenograft formation and metastasis of breast cancer cells in nude mice. Both in vitro and in vivo studies revealed that the 53BP1 expression level was inversely correlated to the function of nuclear factor-kappaB (NF-κB), which contributes to the invasion and metastasis of breast cancer. Importantly, the inhibitory effect of 53BP1 on NF-κB activity was shown to be mediated by the upregulation of miR-146a. Together, our findings demonstrated that 53BP1 has a potent tumor suppressor activity in breast cancer, and it may serve as a novel target for breast cancer prevention and treatment.

Introduction

Breast cancer is the most common female malignancy in the world. In 2012, ~229,060 new breast cancer cases were estimated to be diagnosed in the USA alone, accounting for 29% of all new cancers diagnosed. Although the advent and widespread use of early detection and/or treatment have led to a decrease in death rates, it is estimated that 39,510 women in the USA will die of breast cancer in 2012 (1). Thus, unraveling the molecular mechanisms leading to breast cancer development and progression has become imperative.

Human p53-binding protein-1 (53BP1) was originally identified in a yeast two-hybrid screen (2). Subsequently, it was mapped to chromosome 15q15–q21. The predicted open reading frame of 53BP1 is 6.6 kbp, and its messenger RNA encodes a protein of 1972 residues with a calculated molecular mass of 217 kDa (3). It has been shown that the BRCA1 COOH-terminus domain of 53BP1 binds to the central DNA-binding domain of wild-type p53 and enhances p53-mediated transcriptional activation (2,3). 53BP1 participates in the DNA damage response by responding to ionizing radiation in an ataxia telangiectasia mutated protein-dependent manner and co-localizing with H2AX as well as additional factors at double-stranded DNA breaks (4–6). Experiments using small interfering RNA (siRNA) or gene targeting to knockdown 53BP1 expression have revealed that 53BP1 is required for the accumulation of p53, G2-M checkpoint arrest and intra-S-phase checkpoint in response to DNA damage (7–9). Additionally, 53BP1 co-operates with p53 and suppresses genomic instability through its roles in both cell cycle checkpoints and double-strand break repair (10,11). As the accumulation of genomic alterations resulting from defects in cell cycle checkpoints or DNA repair instability is an important cause of tumorigenesis, 53BP1 is expected to function as a tumor suppressor via the maintenance of genomic stability.

By analyzing the expression of 53BP1 in publicly available gene expression databases, Bouwman et al. revealed a significant correlation between 53BP1 loss and the triple negative phenotype (lacking expression of estrogen receptor and progesterone receptor and without amplification of human epidermal growth factor receptor). Additionally, the survival data demonstrated that the 53BP1-negative tumors had significantly lower metastasis-free survival. Among the triple negative tumors, those that lacked normal 53BP1 staining had worse metastasis-free survival (12). Haffty et al. also found that the homozygous GG genotype, a known common single nucleotide polymorphism in the 53BP1 gene (rs560191), was associated with a significantly higher risk of local relapse upon univariate analysis in a cohort of patients treated with breast-conserving surgery and radiation (13). However, the detailed molecular mechanism of 53BP1 in the development of cancer is still unknown. In this study, we further explored the role of 53BP1 in tumorigenesis and progression of breast cancer.

Materials and methods

Patients and tissue samples

A total of 316 paraffin-embedded breast tissue samples (50 normal breast tissues, 31 usual ductal hyperplasia without atypia (UDH), 17 atypical ductal hyperplasia (ADH) and 29 ductal carcinoma in situ (DCIS) and 189 invasive ductal carcinoma) were obtained from the Department of Pathology, Qilu Hospital, Shandong University between 2007 and 2011. We also collected 39 fresh specimens of breast cancer tissues with matched non-tumor tissue between 2010 and 2011. For the use of these clinical materials for research purposes, prior patient consent and approval from the institutional research ethics committee were obtained. All the diagnoses were made according to the guidelines of the Pathology and Genetics of Tumors of Breast of World Health Organization Classification of Tumors and were made by two pathologists. Clinicopathological classification and staging were determined according to the American Joint Committee on Cancer criteria.

Cell lines and reagents

The breast cancer cell lines MCF-7, T47D, MDA-MB-468 and MDA-MB-231 and the murine fibroblast cell line NIH3T3 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Antibodies against p53, p-p65 (Ser 536), IκB and p-IκB and the murine fibroblast cell line NIH3T3 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Antibodies against p53, p-p65 (Ser 536), IκB and p-IκB were purchased from Cell Signalling Technology (Beverly, MA). Rabbit anti-53BP1 antibody was obtained from Bethyl Laboratories (Montgomery, AL). MiR-146a siRNA and the negative control oligoduplex were obtained from Ambion (Cambridge, MA). The remaining reagents were from Sigma–Aldrich (St. Louis, MO) unless otherwise specified.

Plasmid construction and transfection

The plasmid construction was performed as described previously (14,15). For RNA interference of 53BP1, the sense small hairpin RNA (shRNA) target sequences were as follows: GCCAGGUUCUAGAGGAUAU (9). The pSuper-Neo-GFP (OligoEngine) vector was utilized. The pSuper-Neo-GFP shRNA-53BP1 vector and the empty vector were used to transfect MCF-7 and T47D cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according
to the manufacturer’s protocol to establish pSN-shRNA-53BP1 cell lines (53BP1-KD) and control cell lines (control).

For overexpression of 53BP1 in breast cancer cells, the Addgene plasmid 19836: N-Myc-53BP1 WT pLPC-Puro was employed (16). The expression vector and the empty vector were used to transfect the MDA-MB-231 and MDA-MB-468 cells to establish the 53BP1 overexpression cell line (53BP1-OVE) and the control cell line (control), respectively.

Quantitative reverse-transcription–PCR analysis
Total RNA was extracted with TRIzol reagents according to the manufacturer’s protocol (TaKaRa, Dalian, China). The samples were loaded in quadruplicate, and the results of each sample were normalized to glyceraldehyde 3-phosphate dehydrogenase. For micro RNA (miRNA) analysis, the mirU6 miRNA first-strand complementary DNA synthesis kit and the mirCute mirRNA qPCR detection kit (SYBR Green) from TIANGEN (Beijing, China) were employed. The experiments were repeated in triplicate.

Western blot analysis
Cells were lysed and quantified using the BCA protein assay kit (Merck, Darmstadt, Germany). Nuclear and cytosolic extracts were prepared with a nuclear/cytosol fractionation system according to the manufacturer’s protocol (Beyotime Institute of Biotechnology, Jiangsu, China). Equal amounts of protein were separated on a sodium dodecyl sulfate–polyacrylamide gel and electrotransferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, MA). Signals were detected using enhanced chemiluminescence.

Cell proliferation assay
Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded in a 96-well plate and allowed to attach overnight. Next, 20 µl of the MTT solution (5 mg/ml) was added to each well at the indicated time. The reduction of MTT was quantified by the absorbance using a microplate reader (Bio-Rad, Hercules, CA).

Clonogenic assay
The survival and proliferation potential of cells were assessed using clonogenic assays. Briefly, for the colony forming assay, cells were trypsinized, counted and seeded in six-well plate at 200 cells per well. After incubation for 14 days, colonies were fixed with methanol and stained with crystal violet, and the number of colonies containing >50 cells was scored.

Invasion and migration assay
Invasion and migration assays were performed as described previously (15). Briefly, 1 × 10⁶ cells in serum-free Dulbecco’s modified Eagle’s medium were added to the upper compartment of the chamber. After incubation for 18 hours (migration assay) or 24 h (invasion assay), the non-invasive cells in upper compartment were removed with a cotton swab. The cells in lower compartment of the chamber were counted under a light microscope for at least 10 random visual fields. The migration assay was similar to the invasion assay described above, except that the upper side of the membranes was not coated with the Matrigel.

Immunohistochemistry
The streptavidin–peroxidase–biotin immunohistochemical method was performed to study altered protein expression in paraffin-embedded breast tissues as described previously (17). In brief, sections were microwaved for antigenic retrieval. The primary antibody was incubated with the sections overnight at 4°C; the secondary antibody was from the streptavidin–peroxidase–biotin reagent kit (Zhongshan Biotechnology Company, Beijing, China). After washing, the tissue sections were treated with a biotinylated secondary antibody, followed by further incubation with the streptavidin–horseradish peroxidase complex. Following detection with diaminobenzidine, the sections were counterstained with hematoxylin. For negative controls, the antibody solution was replaced with phosphate-buffered saline.

In vivo tumorigenesis and metastasis assay
The in vivo tumorigenesis and metastasis assay was performed as described previously (14). Briefly, 1 × 10⁶ cells were injected into the right flank of 4- to 5-week-old female severe combined immunodeficient mice. The growth of primary tumors was monitored by measuring tumor diameters. Tumor length (L) and width (W) were measured, and tumor volume was calculated by the equation: volume = (W² × L)/2. Mice were killed 32 days after injection. To produce experimental lung metastasis, 5 × 10⁶ cells were injected into the lateral tail veins of 4- to 5-week-old female severe combined immunodeficient mice. After 5 weeks, the mice were killed under anesthesia. Visible lung surface macrometastatic lesions appeared as white spots. The lungs were collected and fixed in 10% formalin. For tissue morphology evaluation, hematoxylin and eosin (HE) staining was performed on sections from embedded samples. All animal experiments were performed with the approval of Shandong University Animal Care and Use Committee.

Statistical analysis
The results were analyzed using the software SPSS 16.0 (Chicago, IL). Each experiment was conducted three times, and the data were expressed as mean ± standard error of the mean (SEM). A two-tailed Student’s t-test was used to determine the statistical significance. We evaluated the linear-by-linear association from normal, UD, ADH and DCIS to cancer lesion. Bivariate correlations between study variables were calculated by Spearman’s rank correlation coefficients. P values <0.05 were considered statistically significant.

Results
53BP1 was downregulated in breast cancers tissues
Bouwman et al. have shown that 53BP1-negative tumors have significantly lower metastasis-free survival, and the individuals with tumors that lack the normal 53BP1 staining pattern via immunohistochemistry (IHC) have worse metastasis-free survival in breast cancer (12). To further explore the relationship between 53BP1 and breast cancer progression, we first compared the expression levels of 53BP1 in 39 cases of breast cancer tissues and the matched non-tumor tissues using western blotting. The 53BP1 protein level was found to be

![Fig. 1. Expression of 53BP1 in breast cancer tissues. (A) Western blot analysis showed that 53BP1 protein levels were reduced in tumor tissues compared with the matched non-tumor tissues. Results are shown for one of the three independent experiments performed. N, normal tissues; T, tumor tissues. (B) Expression of 53BP1 in normal tissues, UD, ADH, DCIS and breast cancer. P < 0.05. P value represents a linear-by-linear association. (C) Expression of 53BP1 by IHC in breast precancerous lesions and cancer tissue. 53BP1 staining was mainly localized in the nuclei of cells.](image-url)
lower in tumor tissues than in matched non-tumor tissues in 35 cases, to be similar or slightly higher than in the non-tumor tissues in four cases (Figure 1A).

53BP1 level was reduced gradually during the progression from precancerous lesions to cancer

Clinical studies indicated that UDH, ADH and DCIS in the breast are associated with different levels of risk for the subsequent development of invasive carcinoma. As the above data indicated, 53BP1 expression was generally lower in breast cancer tissues compared with non-tumor tissues, although it is unclear whether changes in the expression level of 53BP1 contribute to the progression of breast cancer. We, therefore, investigated the expression of 53BP1 using immunohistochemical staining in a total of 316 paraffin-embedded breast samples including 50 cases of normal breast tissues, 31 cases of UDH, 17 cases of ADH, 29 cases of DCIS and 189 cases of invasive ductal carcinomas. The staining level of 53BP1 was found to drop with increasing stage of breast cancer (Figure 1B). As shown in Figure 1C, 1a positive staining for 53BP1 was defined as brown stain in the nucleus. A specimen was scored as 53BP1 negative if fewer than 10% of the tumor cells showed nuclear staining. Positive 53BP1 staining was identified in most of the normal breast tissues (48 of 50 cases; 96%). In the proliferative lesions, positive 53BP1 staining was detected in 28 of 31 (90.32%) UDH cases, 11 of 17 (64.71%) ADH cases and 9 of 29 (31.03%) DCIS cases. Furthermore, only 31 of 189 (16.40%) invasive breast cancer cases showed positive 53BP1 staining.

53BP1 inhibited proliferation of breast cancer cells

We first detected the protein and messenger RNA levels in breast cancer cell lines MCF-7, T47D, MDA-MB-468 and MDA-MB-231 (Figure 2A). Given that MDA-MB-231 and MDA-MB-468 cells have low endogenous levels of 53BP1, they were used to establish stable cells that constitutively overexpress the 53BP1 protein. As the 53BP1 level was higher in breast cancer MCF-7 and T47D cell lines, we used shRNA to generate 53BP1-knockdown cell models to study the function of 53BP1. The transfection efficiency was confirmed using western blotting and quantitative reverse-transcription–PCR analysis (qRT–PCR). As shown in Figure 2B, the MDA-MB-231 and MDA-MB-468 cells transfected with pLPC-53BP1 plasmid showed significantly increased expression of 53BP1 compared with the control cell lines. In addition, the MCF-7 and T47D cells transfected with shRNA-53BP1 plasmid showed significantly decreased expression of 53BP1 compared with the control cells.

We first examined the effect of 53BP1 expression on cell growth using MDA-MB-231 and MCF-7 human breast cancer cells. The MTT assay showed that upregulation of 53BP1 significantly inhibited the growth of MDA-MB-231 cells, whereas knockdown of 53BP1

Fig. 2. 53BP1 inhibited cell proliferation, invasion and migration of breast cancer cells. (A) Expression of 53BP1 in four breast cancer cell lines was examined using western blotting. β-actin was used as a loading control. (B) The transfection efficiency of 53BP1 was measured by western blot analysis and RT–PCR in cell lines in left and right panels, respectively. (C) Migration and invasion ability were measured using transwell assays in 53BP1-knockdown MCF-7 cells. On the right of each photo is the summary graph for the transwell assay. The cell numbers for migration were 21.35 ± 2.47 versus 49.48 ± 4.58 (P < 0.0001) and invasion were 2.35 ± 0.47 versus 23.48 ± 3.38 (P < 0.0001) in MCF-7 cells. The cell numbers for migration were 93.57 ± 6.39 versus 62.40 ± 4.38 (P < 0.0001) and invasion were 53.51 ± 5.79 versus 34.41 ± 4.16 (P < 0.0001) in MDA-MB-231. All the P-values were <0.0001 for T47D and MDA-MB-468 cells. The data in (C) represent the average cell numbers from at least 10 viewing fields and are presented as the mean ± SEM. All the results are shown for one of the three independent experiments performed.
significantly enhanced the growth of MCF-7 cells (Supplementary Figure 1A). This suggested that 53BP1 could inhibit the growth of breast cancer cells. Next, we performed a clonogenic assay, as shown in Supplementary Figure 1B. Overexpression of 53BP1 reduced colony formation efficiency of MDA-MB-231 and MDA-MB-468, whereas the colony formation efficiency was dramatically increased in the 53BP1-KD MCF-7 and T47D cells compared with the respective control cells.

**53BP1 inhibited invasion and migration of breast cancer cells**

We next assessed whether 53BP1 overexpression/knockdown affects the cell motility of breast cancer cells using a two-chamber assay. 53BP1 overexpression in MDA-MB-231 cells inhibited cell migration and invasion in vitro. Knockdown of 53BP1 in MCF-7 cells enhanced cell migration and invasion (Figure 2C). In addition, knockdown of 53BP1 in T47D cells and overexpression of 53BP1 in MDA-MB-468 cells produced similar results (Figure 2C). Taken together, these results showed that 53BP1 significantly inhibited the invasion and migration of breast cancer cells.

**53BP1 inhibited NF-κB activity through miR-146a**

Constitutive nuclear factor-kappaB (NF-κB) DNA-binding activity has been reported in 65% of primary breast cancers and is also associated with other malignancies (18–20). As NF-κB plays an important role in the progression and metastasis in breast cancers (15,21–23), we speculated that NF-κB activity might be involved in the inhibitory effect exerted by 53BP1 overexpression. Thus, we examined the NF-κB pathway via western blot analysis. As illustrated in Figure 3A, the level of total phosphorylated p65 (p-p65; Ser536) was reduced in 231-53BP1-OVE cells. A lower level of nuclear accumulation of p65 in 231-53BP1-OVE cells was evident by western blot analysis of nuclear and cytosolic extracts (Figure 3B). These results suggest that 53BP1 may have a negative effect on NF-κB activity. Furthermore, the level of IκBα, an NF-κB inhibitor causing p65 cytoplasmic retention, was increased, whereas IκBα phosphorylation at Ser32 (a key signal for IκBα degradation) was decreased in 53BP1-overexpressing MDA-MB-231 cells (Figure 3A). To confirm the inhibitory effect of 53BP1 on the NF-κB pathway in vivo, we also measured the levels of total p65, p-p65, IκBα and p-IκBα in tumor tissues grown in xenotransplants in mice and obtained similar results (Figure 3C). Moreover, there was less nuclear accumulation of p65 and more cytoplasmic p65 in 231-53BP1-OVE tumors, as detected by IHC (Figure 3D). We next determined the expression of NF-κB-regulated genes such as RelB, IL-1β, BCL2A1 and A20 and found that they were downregulated in 231-53BP1-OVE cells when compared with the control cells (Figure 3E).

Furthermore, we performed knockdown of 53BP1 expression in 231-53BP1-OVE via the transfection of the pSN-shRNA-53BP1 interference plasmid. P-p65 in 231-53BP1-OVE was restored to a high level 48 h after transfection of the plasmid (Figure 4A). The invasiveness and motility of the 231-control cells were enhanced (Figure 4B) and the motility of 231-53BP1-OVE cells were also restored in a transwell assay (Figure 4C). We next evaluated the effects of 53BP1 on breast cancer lung metastasis in vivo. The mice were injected into the lateral tail veins with 231-control, 231-53BP1-OVE transfected with shRNA-53BP1 and their respective control plasmid. P-p65 in 231-53BP1-OVE was restored to a high level 48 h after transfection of the plasmid (Figure 4A). The invasiveness and motility of the 231-control cells were enhanced (Figure 4B) and the motility of 231-53BP1-OVE cells were also restored in a transwell assay (Figure 4C). We next evaluated the effects of 53BP1 on breast cancer lung metastasis in vivo. The mice were injected into the lateral tail veins with 231-control, 231-53BP1-OVE transfected with shRNA-53BP1 and their respective control plasmid. As expected, all the lungs from mice injected with shRNA cells (231-53BP1-OVE cells transfected with shRNA-53BP1 interference plasmid) developed extensive metastatic foci that were detectable upon both gross and histological analysis. However, mice injected with control cells (231-53BP1-OVE cells) had no or only a few small metastatic foci that were observable only upon histological analysis (Figure 4D).

There is a growing list of miRNA genes that are involved in a wide range of physiological and pathological processes, including development, differentiation, proliferation, apoptosis and metastasis (24–27). Recently, miR-146a was shown to inhibit both the migration and invasion of cancer cells (28,29), partly through the regulation of NF-κB signaling (28,30). We speculated that miR-146a might act downstream of...
53BP1 inhibited tumorigenesis and metastasis in vivo

To examine the tumor suppressing function of 53BP1 in vivo, 231-control and 231-53BP1-OVE cells were injected into 4- to 5-week-old female nude mice. Tumor volume was measured every 4 days. We found that 231-53BP1-OVE cells have longer latent period than the control cells in tumor formation. Moreover, the tumors formed by 231-53BP1-OVE cells were smaller than those by control cells (Figure 6A). These results indicated that 53BP1 could inhibit breast cancer cell xenograft formation and growth in vivo. Interestingly, when the tissues were examined after HE staining, the tumors formed by 231-53BP1-OVE cells appeared to be more compact when compared with control tumors, in which the tumor cells were scattered and appeared to be more diffusive (Figure 6B), which may be attributable to the inhibitory effect of 53BP1 on the invasion and metastatic ability of breast cancer cells.

We further evaluated the in vivo effects of 53BP1 on breast cancer lung metastasis. An equal number of 231-control and 231-53BP1-OVE cells were injected into the lateral tail veins of nude mice. Five weeks later, all 11 lungs from control mice exhibited numerous white spots on the surface that represented macrometastases; such spots were absent in the lungs from the 231-53BP1-OVE mice (Figure 6C). For tissue morphology evaluation, HE staining was performed on sections from embedded samples. As expected, the lungs from mice injected with control cells developed extensive metastatic foci that were detectable by both gross and histological analysis. However, mice injected with 231-53BP1-OVE cells showed no or only a few small metastatic foci upon histological analysis only (Figure 6C).

Discussion

53BP1 is reported to play an important role in both cell cycle checkpoints and DNA repair activity. Researchers have reported a decrease or loss of 53BP1 expression in various tumors and that these tumors conveyed a significant lower disease-free survival in cancer patients, including those with breast cancer (12,31). Hong et al. (32) showed
Fig. 5. 53BP1 inhibited metastasis ability through miR-146a. (A) 53BP1 overexpression upregulated miR-146a, whereas 53BP1 knockdown downregulated miR-146a. (B) The miR-146a knockdown was detected by qRT–PCR in 231-control and 231-53BP1-OVE cells following transfection with the miR-146a siRNA or the negative control. (C) Migration ability was measured using transwell chamber assays after knockdown of miR-146a. (D) The summary graphs for the migration and invasion assay \( (P = 0.001 \text{ and } P = 0.004, \text{ respectively}) \). (E) Invasion ability was measured using transwell chamber assays after knockdown of miR-146a. (F) The summary graphs for the migration and invasion assay \( (P < 0.001 \text{ and } P = 0.009, \text{ respectively}) \). In C–F, the data represent the average cell number from at least 10 viewing fields and are presented as the mean ± SEM. (G) The levels of p65 and p-p65 were determined by western blotting following the knockdown of miR-146a. Results are shown for one of the three independent experiments performed.
that 53BP1 suppresses tumor growth of ovarian cancer cells through inhibiting the Akt pathway. These data suggested that 53BP1 was a potential tumor suppressor in breast cancer.

To further explore the role of 53BP1 in tumor suppression in breast cancer, we first compared the expression levels of 53BP1 in breast cancer tissues and the matched non-tumor tissues using western blot analysis. A decreased 53BP1 protein level was found in tumor lesions compared with matched non-tumor tissues. Next, we determined the expression levels of 53BP1 at different stages during the progression of breast cancer. Our results demonstrate that 53BP1 expression level is higher in normal tissues, but upon the intraductal proliferative lesion progression (UDH, ADH and DCIS), it became lower gradually. This suggested that 53BP1 might represent a novel indicator for the carcinogenesis of breast lesions and that the loss of 53BP1 function might promote the progression of breast cancer lesions.

To further verify the function of 53BP1, we manipulated the expression level of 53BP1 in breast cancer cells with RNA interference or with ectopic expression. We found that 53BP1 significantly inhibited growth of breast cancer cells in vitro and in vivo. In line with clinical data, cells overexpressing 53BP1, the 231-53BP1-OVE cells, presented a decreased invasion and migration potential compared with the control cells. In vivo, the tumors formed by the control cells were scattered and probably to migrate to the nearby tissues, whereas those of the 231-53BP1-OVE cells appeared to be more compacted. Additionally, by using breast cancer lung metastasis model, we found that 231-53BP1-OVE cells have greatly reduced ability to form metastatic foci in the lungs than in control cells. When 53BP1 was knockdown in 231-53BP1-OVE cells, the migration and invasion were turned back to higher level. Also, the metastasis to lung in vivo was increased when the 53BP1 was knocked down. This further implied that 53BP1 could inhibit the metastasis of breast cancer in vivo. However, we should point out that the 231-53BP1-OVE...

**Fig. 6.** 53BP1 overexpression inhibited the tumorigenic and metastatic capabilities of MDA-MB-231 cells. (A) Growth curves of mammary tumors after the injection of the control and 53BP1-overexpressing MDA-MB-231 cells in mice (P < 0.001). The inset depicts a representative picture of the mammary tumor from the mice. Each group consisted of eight mice. Error bars represent ±SEM. (B) The tissues harvested from the mice were stained by HE. (C) Representative pictures of lungs and HE-stained sections of the representative lungs harvested at necropsy from mice injected with control and 53BP1-overexpressing MDA-MB-231 cells. The right panel is the enlarged picture marked in the box of its middle panel. Original magnification: ×40 (middle) and ×100 (right). Each group consisted of 11 mice.
tumors had a much slower growth rate than controls, which might lead to an underestimation of their ability to metastasize.

Our results also established a link between 53BP1 and NF-κB signaling. Both in vitro and in vivo assays showed that when the 53BP1 is overexpressed, the level of phosphorylated p65 was decreased, and the NF-κB target genes were downregulated. This suggests that NF-κB pathway is negatively regulated by 53BP1 in breast cancer. Moreover, we found that 53BP1 positively regulates the expression of mir-146a. Mir-146a was significantly upregulated upon the introduction of 53BP1 into the MDA-MB-231 cells, whereas mir-146a was significantly downregulated in 53BP1-knockdown MCF-7 cells. Knockdown of mir-146a by mir-146a siRNA led to a significant increase in the level of phosphorylated p65, and in the invasion and migration ability of the 53BP1-OVE cells. Consistent with the previous findings, we found that miR-146a could inhibit both the migration and invasion of cancer cells through NF-κB signaling. Therefore, 53BP1 may inhibit the ability of breast cancer cells to metastasize, at least partly, through the miR-146a-mediated NF-κB pathway. Paik et al. (33) have reported that mir-146a downregulated NF-κB activity via inhibiting TRAF6 and IRAK1. Future work should be conducted to unravel how mir-146a on NF-κB is also mediated by TRAF6 and IRAK1.

In summary, we found a significant association between loss of 53BP1 and the progression of breast cancer. 53BP1 expression was generally lower in breast cancer lesions than in the matched non-tumor tissues, and the expression level decreased with the progression of the breast cancer. Our in vitro and in vivo findings demonstrate that 53BP1 plays an important role in the inhibition of cell growth, invasion and metastasis, partially via the inhibition of NF-κB through mir-146a. Further investigation is warranted to determine whether targeting 53BP1 may be an effective novel target in breast cancer prevention and treatment.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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