RhoBTB3 regulates proliferation and invasion of breast cancer cells via Col1A1

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Abstract

Background: Breast cancer is the leading cause of cancer-related death in women worldwide, despite medical and technological advancements. The RhoBTB family consists of three isoforms: RhoBTB1, RhoBTB2, and RhoBTB3. RhoBTB1 and RhoBTB2, have been proposed as tumor suppressors in breast cancer. However, the roles of RhoBTB3 proteins are unknown in breast cancer.

Methods: Bioinformatics analysis, including Oncomine, cBioportal, was used to evaluate the potential functions and prognostic values of RhoBTB3 and Col1A1 in breast cancer. qRT-PCR analysis and immunoblotting assay were performed to investigate relevant expression. Functional experiments including proliferation assay, invasion assay, and flow cytometry assay were conducted to determine the role of RhoBTB3 and Col1A1 in breast cancer cells.

Results: RhoBTB3 mRNA levels were significantly up-regulated in breast cancer tissues as compared to in adjacent normal tissues. Moreover, RhoBTB3 expression was found to be associated with Col1A1 expression. Decreasing RhoBTB3 expression may lead to decreases in the proliferative and invasive properties of breast cancer cells. Further, Col1A1 knockdown in breast cancer cells limited the proliferative and invasive ability of cancer cells.

Conclusion: Knockdown of RhoBTB3 may exert inhibit the proliferation, migration, and metastasis of breast cancer cells by repressing the expression of COL1A1, providing a novel therapeutic strategy for treating breast cancer.

Keywords: RhoBTB3, Col1A1, human breast cancer

Background
Breast cancer is the most common form of cancer and leading cause of cancer-related death for women worldwide. Previous studies revealed that hormones such as estrogen and progestin as well as genetic mutation and various other molecules can cause malignancy in breast tumors [1, 2]. Despite significant advances in cancer prevention and targeted chemotherapy, the incidence of breast cancer and associated mortality continue to increase [3]. Therefore, more effective therapeutic targets are required to optimize the clinical management of breast cancer.

The RhoBTB (Rho-related Broad-complex, Tramtrack, and Bric-à-brac) family consists of three isoforms: RhoBTB1, RhoBTB2, and RhoBTB3. These molecules have a unique domain architecture in which a GTPase domain is followed by a proline-rich region, tandem of 2 BTB domains, and conserved C-terminal region [4, 5]. Two of the three RhoBTB proteins, RhoBTB1 and RhoBTB2, differ substantially from RhoBTB3 [5]. However, their precise functions and underlying mechanisms in suppressing breast cancer are poorly understood.

Recently, collagen type I alpha 1 (Col1A1) was reported to be associated with a variety of cancers, and its overexpression was observed in tissues and cells of breast, lung, and renal cancers [6-8]. Moreover, increased collagen deposition is associated with breast cancer cell proliferation and invasion [9, 10]. However, the regulatory mechanism of Col1A1 in breast cancer remains unclear despite numerous recent investigations in human oncology.

In this study, we examined whether RhoBTB3 regulates collagen synthesis and secretion in breast cancer. We show that increased mRNA levels of RhoBTB3 and Col1A1 are associated with poor prognosis of patients with breast cancer. Knockdown of RhoBTB3 regulates breast cancer cell proliferation and invasion, accompanied by
reduced Col1A1. Furthermore, expression of RhoBTB3 and Col1A1 is significantly correlated in breast cancer. These results suggest that RhoBTB3 regulates breast cancer progression by controlling collagen deposition and may serve as a therapeutic target for breast cancer.

**Materials and methods**

**Plasmid and siRNA**

pCS2-FLAG-RhoBTB3 construct was provided by Suzanne R Pfeffer lab (Stanford University School of Medicine, Stanford, CA, USA). Col1A1 ORF-containing plasmid pECFP-N2-Col1A1 was purchased from Addgene (plasmid 66603, Watertown, MA, USA). Control (siRNA pool 1, D-001206-13-05), human specific RhoBTB3, and Col1A1 siRNAs (M-010224-02, M-015890-02, siGENOME SMART pools) were purchased from Dharmacon (Lafayette, CO, USA).

**Cell culture and transfection**

Human breast cancer cell line (MDA-MB-231) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in high-glucose Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C in a humidified 5% CO₂ incubator. Plasmid DNA and siRNA were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.

**Immunoblotting**

For immunoblotting analysis, control and siRhoBTB3 cells were trypsinized and
counted; equal amounts of conditioned medium (normalized to cell number) were precipitated using PEG8000 (Promega, Madison, WI, USA). Equal amounts of protein were electrophoresed on 6%–10% SDS-PAGE, transferred to PVDF membranes (Millipore, Burlington, Massachusetts, MA) and probed with Anti-Collagen Alpha-1(I) Chain Carboxy-Telopeptide antibody (LF-68, 1:3000; Kerafast, Boston, MA, USA).

**Immunofluorescence microscopy**

Cells were seeded in a 6-well plate with acid-washed glass coverslips. After 24 h, the cells were fixed with 4% paraformaldehyde in DPBS for 30 min, washed five times with PBS, and permeabilized with 0.1% Triton X-100 in DPBS for 15 min at room temperature (RT) or 25 °C. Thereafter, the cells were incubated with blocking buffer (0.5% BSA in PBS) for 30 min at RT, followed by 1 h incubation at RT with rabbit anti-LF68 (1:500; prepared in 0.5% BSA in DPBS) and Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (1:500, Molecular Probes, Invitrogen). The cells were subsequently subjected to five washes with PBS. Images were obtained using Axio Observer Z1 fluorescence microscope (Carl Zeiss, Germany) and merged using the Zeiss Zen 2.3 software.

**Real-time PCR with reverse transcription**

Total RNA was isolated using RNeasy Mini Kit (QIAGEN, Frederic, MD, USA) according to the manufacturer’s protocol. Reverse transcription was performed using 1 mg of the total RNA as template and Super-Script™ III Reverse Transcriptase (Invitrogen). qRT-PCR was performed in triplicates on a LightCycler 480 (Roche, Basel, Switzerland) using LightCycler480 SYBR Green I Master Mix (Roche) and the
following primers: human Col1A1, 5′-GTGTTGTGCGTGSCG-3′ and 5′-TCGGTGGGTGACTCT-3′; human RhoBTB3, 5′-CTGGTGATCTTTAGGTGGT-3′ and 5′-GTGCTGGTGGGATGTTG-3′; human GAPDH, 5′-CTCCTCCACCTTTGACGC-3′ and 5′-CCACCACCTGTGTGCTGT-3′. Expression of the housekeeping gene GAPDH was used to normalize the data.

**Cell cycle analysis**

Cells were harvested, resuspended, and fixed with 70% (v/v) ice-cold ethanol overnight. Fixed cells were washed twice with ice-cold PBS and centrifuged at 300 g for 5 min. The cells were then incubated for 30 min at RT with staining solution (0.1 mg/mL RNase A, 50 μg/mL propidium iodide). Samples were analyzed using an FACS Calibur cytometer (BD Biosciences, San Jose, CA, USA).

**Proliferation assay**

Cells were transfected with siRhoBTB3, siCol1A1, and/or Col1A1 plasmid DNA for 24 h, trypsinized, and resuspended in medium. After 48 h, the cells were seeded in 96-well plates at a density of 4 × 10³ cells/well. After 72 h of treatment, a mixture of CyQUANT NF Cell proliferation dye reagent and deliverer (Invitrogen) was added to the wells, and the plates were incubated at 37 °C for 30 min. Fluorescence intensity was measured as the ratio of fluorescence at 530 nm to that at 485 nm.

**Invasion assay**

Transwell chambers (Corning, NY, USA) were coated with Matrigel Basement Membrane Matrix (BD Biosciences). Cells were suspended in serum-free medium and
seeded in the upper chamber at a density of $2 \times 10^3$ cells/well, whereas serum-containing medium was placed in the lower chamber. After incubating for 24 h, the cells penetrating through the pores were stained with Diff-Quik staining solution (Sysmex Co., Kobe, Japan) and observed under a microscope.

**Statistical analyses**

To assess expression levels of RhoBTB3 and Col1A1 in human breast cancer, we retrieved data profiles from Oncomine ([www.oncomine.org](http://www.oncomine.org)). We analyzed the expression levels in invasive breast carcinoma, invasive ductal breast carcinoma, and ductal breast carcinoma in situ epithelia. We used cBioPortal (Breast Invasive Carcinoma, TCGA, Nature 2012) to analyze correlation between the expression levels of RhoBTB3 and Col1A1([www.cbioportal.org](http://www.cbioportal.org)). All experiments were repeated at least three times. Results are reported as mean ± SEM (standard error of mean). Significance of difference was assessed by independent Student's t-test. Value of $P < 0.05$ was considered statistically significant.

**Results**

**Upregulation of RhoBTB3 in breast cancer tissue**

To investigate whether the expression levels of RhoBTB3 are associated with breast cancer tissue, we assessed the mRNA expression of RhoBTB3 in TCGA human breast cancer using the Oncomine database. RhoBTB3 expression was significantly upregulated in invasive breast carcinoma ($P < 0.05$) and invasive ductal breast carcinoma ($P < 0.001$) as compared to in normal breast tissue, indicating that
RhoBTB3 is an oncogene in breast cancer (Fig. 1a). Furthermore, we examined whether RhoBTB3 expression is associated with clinical outcomes in patients with human breast cancer. Kaplan-Meier survival analysis showed that breast cancer patients with high RhoBTB3 expression had significant shorter relapse-free survival (Fig. 1b). Thus, our systematic analysis based on a bioinformatics database may help researchers determine the role of RhoBTB3 in breast cancer and can be targeted as potential oncogenic markers for breast cancer treatment.

RhoBTB3 modulates breast cancer cell growth and invasion

To assess the role of RhoBTB3 in regulating the proliferation and invasive ability of breast cancer cells, we transfected MDA-MB-231 cells with siControl and siRhoBTB3. Cell proliferation was significantly decreased in siRhoBTB3-transfected cells as compared to in control cells (Fig. 2a–b). Further, we evaluated the effect of siRhoBTB3 on cell cycle progression. As shown in Fig. 2c, a larger number of siRhoBTB3-transfected cells was observed in S and G2/M phases as compared to the control cells. These results suggest that the proliferation-promoting function of RhoBTB3 is mediated by promoting S and G2/M phase transitions in breast cancer cells. In addition, we examined whether RhoBTB3 affects the invasive ability of breast cancer cells. As shown in Fig. 2d, the invasive ability of RhoBTB3 knockdown cells was significantly reduced as compared to that of control cells. Taken together, our findings reveal the role of RhoBTB3 in regulating the proliferation and invasive ability of breast cancer cells.

Col1A1 is high expressed in breast cancer tissue, while knockdown of Col1A1
inhibit breast cancer cell growth and invasion

Similar to RhoBTB3, we retrieved the expression profiles of Col1A1 in human breast cancer (Oncomine database). Col1A1 was found to be upregulated in breast cancer tissues compared to in normal tissues. These data are consistent with those of previously published studies on Col1A1 expression in breast cancer cells (Fig. 3a). Furthermore, Kaplan-Meier survival analysis showed that patients with breast cancer with high RhoBTB3 expression had significant shorter relapse-free survival (Fig. 3b). These results may help researchers determine the role of Col1A1 in breast cancer and identify potential oncogenic markers for breast cancer treatment.

RhoBTB3 reduces breast cancer cell growth and invasion by down regulating Col1A1

We investigated whether RhoBTB3 regulates proliferation and invasion of breast cancer cells via Col1A1. RhoBTB3 interference models were constructed. As shown in Fig. 4a, RhoBTB3 expression in breast cancer cells was decreased significantly following transfection with siRhoBTB3 (Fig. 4a). To identify the effects of Col1A1 on the proliferation and invasion of breast cancer cells, we performed various assays. The proliferation assay revealed that knockdown of RhoBTB3 significantly decreased the growth rate of breast cancer cells (Fig. 4b). Additionally, reduced invasive ability was observed in Col1A1 knockdown cells as compared to in control cells. The number of invaded Col1A1 knockdown cells decreased to 60% of that of control cells when MDA-MB-231 cells were used (Fig. 4c). Furthermore, we examined the effect of RhoBTB3 depletion on collagen synthesis and secretion. Interestingly, qPCR and immunoblotting analysis showed that knockdown of RhoBTB3 in MDA-MB-231 cells
reduced mRNA and secreted Col1A1 levels (Fig. 4d–e). In addition, deposition of collagen I was reduced in RhoBTB3-silenced MDA-MB-231 cells compared to in control MDA-MB-231 cells, as indicated via immunofluorescence microscopy (Fig. 4f). Indeed, RhoBTB3 inhibition led to decreased cell proliferation as compared to that of control cells, which was rescued upon Col1A1 overexpression (Fig. 5a). Consistent with the invasion data, RhoBTB3-depleted cells exhibited a reduced invasive ability as compared to control cells. This reduction was rescued by simultaneous Col1A1 overexpression (Fig. 5b). These results suggest that RhoBTB3 promotes cancer growth and invasion by regulating collagen deposition. Notably, expression of RhoBTB3 correlated positively with the expression of Col1A1 according to analysis using cBioPortal (Breast Invasive Carcinoma, TCGA) (Fig. 5c). In summary, these data indicate that RhoBTB3 expression plays a critical role in human breast cancer, possibly by modulating collagen-associated cancer development and progression.

Discussion
Genes encoding RhoBTB proteins exhibit ubiquitous but tissue-differential expression [5, 11, 12]. RhoBTB1 and RhoBTB2 have been regarded as tumor suppressors. Their expression is significantly decreased in breast cancer tissues as compared to normal tissues [13-16]. McKinnon et al. reported that loss of RhoBTB1 in developing cancer reduces METTL7B expression, thereby promoting the loss of normal epithelial polarity through reduced METTL7B expression and contributes to the switch to an invasive phenotype [14]. RhoBTB2 (also known as deleted in breast cancer, DBC2) was proposed as a candidate tumor suppressor gene, as its expression in breast cancer cells lacking RhoBTB2 transcripts caused growth inhibition [15, 16]. In contrast to the
tumor-suppressive role of RhoBTB1 and RhoBTB2 in human breast cancer, our
findings demonstrated an oncogenic role for RhoBTB3 in breast cancers. The
structural differences between RhoBTB1/2 and RhoBTB3 may have led to these
contradictory results. The RhoBTB family has a common domain architecture.
RhoBTB1 and RhoBTB2 are highly similar, whereas RhoBTB3 is the most divergent
member. Interestingly, the RhoGTPase domain of RhoBTB3 binds and hydrolyses ATP,
whereas that of RhoBTB1/2 binds GTP. Moreover, only RhoBTB3 bears an
isoprenylation CAAX motif that is typical of classical RhoGTPases in the C-terminal
region [5, 17]. The CAAX motif is widely involved in global cellular functions, such as
proliferation and differentiation [5, 11, 18]. As an important modulator of biological
activity, signal transduction via protein prenylation is a crucial step for most CAAX motif
functions, particularly for anchoring these motifs to the cellular membrane system [4,
12, 19]. Therefore, we suggest that the oncogenic function of RhoBTB3 in breast
cancer is possibly an outcome of the protein’s structural differences with the isoforms
RhoBTB1/2.

Col1A1, a major component of the extracellular matrix in the tumor
microenvironment, plays a major role in cancer development and progression. It has
been reported that Col1A1 is highly expressed in the cytoplasm in breast cancer cells
compared to in normal cells [20, 21]. In addition, Liu et al. demonstrated that
downregulation of Col1A1 reduced breast cancer growth and metastasis, whereas its
upregulation significantly increased breast cancer proliferation, migration, and
invasion [22]. These studies indicate that increased Col1A1 expression promotes
breast cancer development and progression by enhancing tumor growth and invasion.
However, regulation of Col1A1 expression in breast cancer cells is unclear. Our results
show that mRNA expression of Col1A1 is regulated by RhoBTB3. The Rho signal has been reported to be involved in regulating type I collagen synthesis. ROCK signaling, which involves a member of the Rho family, regulates Col1A1 synthesis by nuclear localization of MRTF-A in breast cancer cells [12-14]. Similarly, we suggest that RhoBTB3 regulates Col1A1 expression through transcriptional regulation.

**Conclusion**

In summary, this study provides strong evidence of the oncogenic effects of RhoBTB3 mediated by regulating Col1A1 in breast cancer. We observed significant upregulation of RhoBTB3 expression in breast cancer tissue specimens compared to that in the corresponding normal tissue specimens. Furthermore, knockdown of RhoBTB3 reduced breast cancer cell proliferative and invasive properties. Additionally, knockdown of Col1A1 in breast cancer cells led to extremely decreased proliferation and invasion, similar to the results obtained for RhoBTB3 downregulation. These results suggest the potential application of RhoBTB3 as a diagnostic and therapeutic target in breast cancers. Further research will seek to identify the transcription factor that regulate Col1A1 expression by interacting with RhoBTB3.

**List of abbreviations**

RhoBTB: Rho-related Broad-complex, Tramtrack, and Bric-à-brac

Col1A1: Collagen type I alpha 1

TCGA: The Cancer Genome Atlas
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Not applicable.

Authors’ contributions

K.K. and Y.-J.K. designed the experiments. K.K. performed the experiments. K.K. and Y.-J.K. wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Please contact author for data requests.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Declarations

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

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**Figure legend**

**Figure 1. Loss of RhoBTB3 suppresses growth and invasion of breast cancer cells.**

(a) The box plot comparing specific RhoBTB3 expression in normal (left plot) and cancer tissue (right plot) was derived from the Oncomine database. The analysis was shown in invasive breast carcinoma and invasive ductal breast carcinoma relative to in normal breast tissue. (b) The survival curve comparing patients with high (red) and low (black) expression of RhoBTB3 in breast cancer was plotted from the Kaplan Meier-plotter.

**Figure 2. Loss of RhoBTB3 suppresses growth and invasion of breast cancer cells.**

(a) qRT-PCR analysis of RhoBTB3 expression after RhoBTB3 knockdown. (b) Cell proliferation assay, (c) micrograph and cell invasion assay, and (d) cell cycle analysis (cell number and cell cycle distribution assays) after RhoBTB3 knockdown. Data are representative of three independent experiments. Error bars represent ± SEM. *P < 0.05, **P < 0.01. Scale bar, 200 μm.

**Figure 3. RhoBTB3 is associated with collagen expression in breast cancer**
(a) The box plot comparing specific Col1A1 expression in normal (left plot) and cancer tissue (right plot) was derived from the Oncomine database. The analysis was shown in invasive breast carcinoma and invasive ductal breast carcinoma relative to in normal breast tissue. (b) The survival curve comparing patients with high (red) and low (black) expression of Col1A1 in breast cancer was plotted from the Kaplan Meier-plotter.

Figure 4. RhoBTB3 is associated with collagen expression in breast cancer
(a) Col1a1 qRT-PCR analysis after col1a1 knockdown. (b) Cell proliferation assay, (c) micrograph and cell invasion assay. (d) Col1a1 qRT-PCR analysis after RhoBTB3 knockdown. (e) Immunoblot analysis of Col1A1 in conditioned medium after RhoBTB3 knockdown. (f) Immunofluorescence staining of Col1A1 (green) and DAPI (blue) after RhoBTB3 knockdown. Error bars represent ± SEM. * P < 0.05, ** P < 0.01. Scale bar, 100 μm.

Figure 5. RhoBTB3 affects proliferation and invasion in an Col1a1-dependent manner. (a-b) Cell proliferation and invasion assays after Col1a1 knockdown. (c) Scatterplot of correlated mRNA levels between RhoBTB3 and Col1A1 in normal and malignant breast tissues. Data are representative of three independent experiments. Error bars represent ± SEM. * P < 0.05, ** P < 0.01.