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BST-2 is a potential activator of invasion and migration in tamoxifen-resistant breast cancer cells

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ABSTRACT

Bone marrow stromal cell antigen 2 (BST-2) is a type II transmembrane protein that is known to be a therapeutic target in several types of cancer. However, despite its clinical importance, the roles of BST-2 expression have remained elusive. Here, we found that BST-2 expression is up-regulated in tamoxifen-resistant MCF-7 human breast cancer (TRM-7) cells, resulting in enhanced invasiveness and migration. Matrigel and wound healing assays also showed that overexpression of BST-2 increased invasion and migration in MCF-7 cells, whereas invasion and migration were decreased by the silencing of BST-2 in TRM-7 cells. In addition, B16F10 cells expressing BST-2 showed increased metastatic melanoma nodule growth in a lung metastasis mouse model. Furthermore, BST-2 expression and promoter activity were regulated by activated signal transducer and activator of transcription 3 (STAT3). Taken together, our results indicate that BST-2 is an important factor in the invasiveness and motility of tamoxifen-resistant breast cancer cells, and that its expression and activity are regulated by activated STAT3. Therefore, regulation of BST-2 is a potential therapeutic target for tamoxifen-resistant breast cancer.

1. Introduction

Tamoxifen resistance is a major challenge in the management of breast cancer patients. Despite an initial response to therapy, the majority of patients ultimately relapse and present with disease progression. We previously reported that upon acquisition of tamoxifen resistance, breast cancer cells increase their motile and invasive behavior *in vitro* [1]. In spite of evidence that tamoxifen resistance promotes their motility and invasion, the molecular mechanisms and novel target genes in the cellular signaling pathway have not been fully investigated.

BST-2 (also referred to as CD317 and HM1.24) was originally identified as a surface antigen that is preferentially expressed in terminally differentiated human B cells. It is known to be involved in pre-B cell growth and development of plasma cells in multiple myeloma [2]. We previously reported that IFN-γ-induced BST-2 expression is associated with the recruitment of monocytes to sites of inflammation in human endothelial cells [3]. Furthermore, it was recently observed that BST-2 is overexpressed in several cell types of solid tumors [4], which exhibited invasive or drug-resistant phenotypes [5–7]. However, the expression status and role of BST-2 in solid tumors are not well known. Therefore, the crucial role of BST-2 expression in breast cancer should be clarified.

The aim of this study was to determine whether BST-2 is involved in invasive behaviors in TRM-7 cells selected by long-term culture with tamoxifen. To achieve this, we determined BST-2 expression and invasive properties in *in vitro* and *in vivo* models. We found that BST-2 is expressed in TRM-7 cells, and that this expression enhanced invasive and motile behavior. BST-2 expression and promoter activity were regulated by activated STAT3.

2. Materials and methods

2.1. Cell culture

The human breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Tamoxifen-resistant MCF-7 (TRM-7) cells were established accord-

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ing to the previously described method [1,8]. Cells were cultured at 37 °C in 5% CO_2/95% air in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Lonza Inc., US-Allendale, NJ, USA).

2.2. Immunoblotting

Cells were lysed in a lysis solution containing protease and phosphatase inhibitor cocktails (Roche, Switzerland). After incubation for 20 min on ice, insoluble debris was removed by centrifugation at 13,000 rpm for 20 min at 4 °C. Total protein was resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes (GE Healthcare, Pittsburgh, PA, USA). The membranes were then probed with antibodies against α-tubulin (Thermo Fisher Scientific, Fremont, CA, USA), phospho-tyrosine STAT3 (Cell Signaling Technology, Danvers, MA, USA) and purified polyclonal BST-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and visualized using the SuperSignal West Femto Chemiluminescent Substate (Thermo Fisher Scientific).

2.3. Quantitative real-time reverse transecription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated and cDNA was synthesized using a QuantiTech Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative reverse-transcription PCR was performed using the QuantiFast SYBR Green PCR master mix (Qiagen). Quantitative reverse-transcription PCR with an Applied Biosystems 7300 Real-time PCR System (Life Technologies, Carlsbad, CA, USA). The raw data were analyzed by comparative Ct quantification. Primers against BST-2 and GAPDH were obtained from Qiagen.

2.4. Transfection and luciferase assay

BST-2 reporter construct (pGL3-BST-2) was prepared by inserting a region of the human BST-2 promoter into the pGL3 basic luciferase reporter plasmid. Wild-type STAT3 (wtSTAT3) and point-mutated STAT3 (Y705F STAT3) was cloned into the pCMV vector (Invitrogen, Carlsbad, CA, USA). MCF-7 cells were co-transfected with BST-2 reporter construct and either empty, wtSTAT3 or Y705F STAT3 using LTX transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Luciferase assay was performed as described elsewhere [8].

2.5. Immunofluorescence

Cells grown on 35 mm lysine-coated, glass-bottomed culture dishes were fixed with freshly prepared 4% parapormaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. Subsequently, cells were blocked with 5% normal serum in PBS for 1 h and then incubated with anti-BST-2 antibody at 4 °C overnight. They were incubated with appropriate secondary antibodies. Stained cells were mounted in VECTASHIELD mounting media with

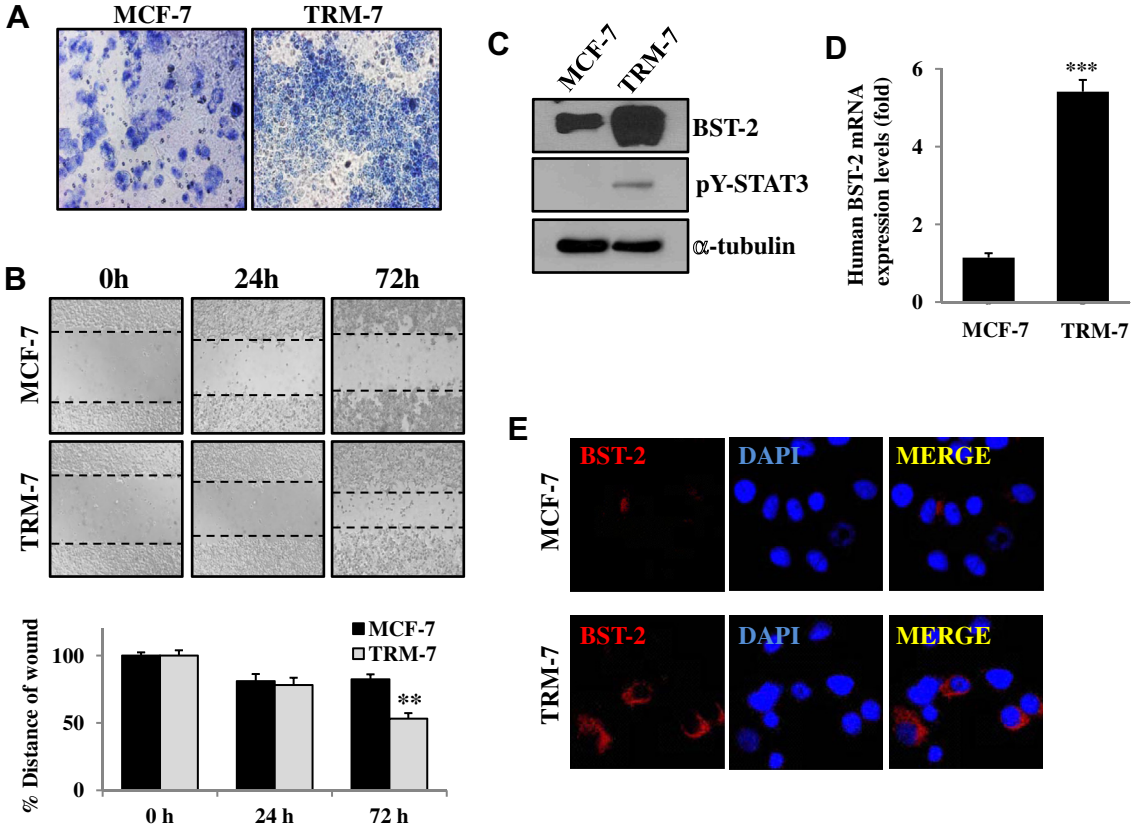

Fig. 1. Correlation between metastatic potential and BST-2 expression in TRM-7 cells. (A) Cells were seeded onto a Matrigel-coated porous membrane and incubated for 24 h. Invading cells were fixed and subsequently stained using Diff-Quik solution. The original photographs were taken at 10× magnification. (B) Cells were seeded into six-well plates and scratched with a 200-μl pipette tip upon reaching 80–90% confluence. Wound closure was monitored (4× magnification). *Columns*, means of triplicate samples; *bars*, S.D. $^{**}P < 0.01$. (C) BST-2 and phosphorylated STAT3 protein levels were evaluated by Western Blotting. (D) BST-2 mRNA levels were determined by quantitative real-time PCR. The level of each mRNA was normalized to that of GAPDH. *Columns*, means of triplicate samples; *bars*, S.D. $^{**}P < 0.01$, $^{***}P < 0.001$. (E) Cells were stained with an anti-BST-2 antibody (red) and nuclei were stained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

DAPI (Vector Laboratories, Burlingame, CA, USA) and visualized under a confocal laser scanning microscope (LSM 510; Carl Zeiss, Gottingen, Germany).

2.6. Cell invasion assay

Invasiveness was assessed using a Boyden chamber system (Neuro Probe, Gaithersburg, MD, USA) with a polycarbonate membrane having a pore size of 8 μM separating the two chambers. The upper chamber was coated on ice with 100 μl of growth factor-recued Matrigel (BD Biosciences, San Jose, CA, USA) containing 0.5 mg/ml protein and incubated at 37 °C for at least 4 – 5 h to allow gelling. The lower chamber was filled with 600 ml of DMEM containing 10% fetal bovine serum (FBS) and 5 mg/ml fibronectin. Cells were loaded into the upper chamber and incubated at 37 °C for 24 h in an atmosphere 95% air and 5% CO_2. The upper medium was then removed, and the membranes were rinsed in PBS and fixed. The membranes were subsequently stained with Diff-Quik solution (Sysmex Corp. Kobe, Japan) and then washed twice with PBS. Attached cells onto the membrane were photographed.

2.7. Wound healing assay

Cells were seeded into 12-well plate. After the cells grew to confluence, wounds were made by scratch with sterile pipette tips. Cells were washed with PBS and refreshed with 10% FBS. After overnight incubation at 37 °C, the cells were fixed and photographed.

2.8. Animal study

B16F10 melanoma cells were seeded at 1×10^5 cells per well in 12-well plate and cultured for 1 day. Cells were then transfected with empty vector or BST-2 construct using a lipofectamin 2000 transfection reagent (Invitrogen), following the manufacturer's instructions. Transfected cells were selected incubation with 1000 μg/ml G418 for 4 weeks, and the resistant clones were isolated and cultured. B16F10 cells expressed BST-2 construct (1×10^6 cells in 100 μL PBS) were intravenously injected into the tail veins. Lung specimens were collected on day 14 to detect metastatic colonies.

2.9. Statistical analysis

Data are presented as means ± standard error of means (S.E.M.) of three independent experiments. Statistical analysis of differences among groups was determined by Student's *t*-test, and differences were considered statistically significant at a level of $p < 0.001$, $p < 0.01$ or $p < 0.05$.

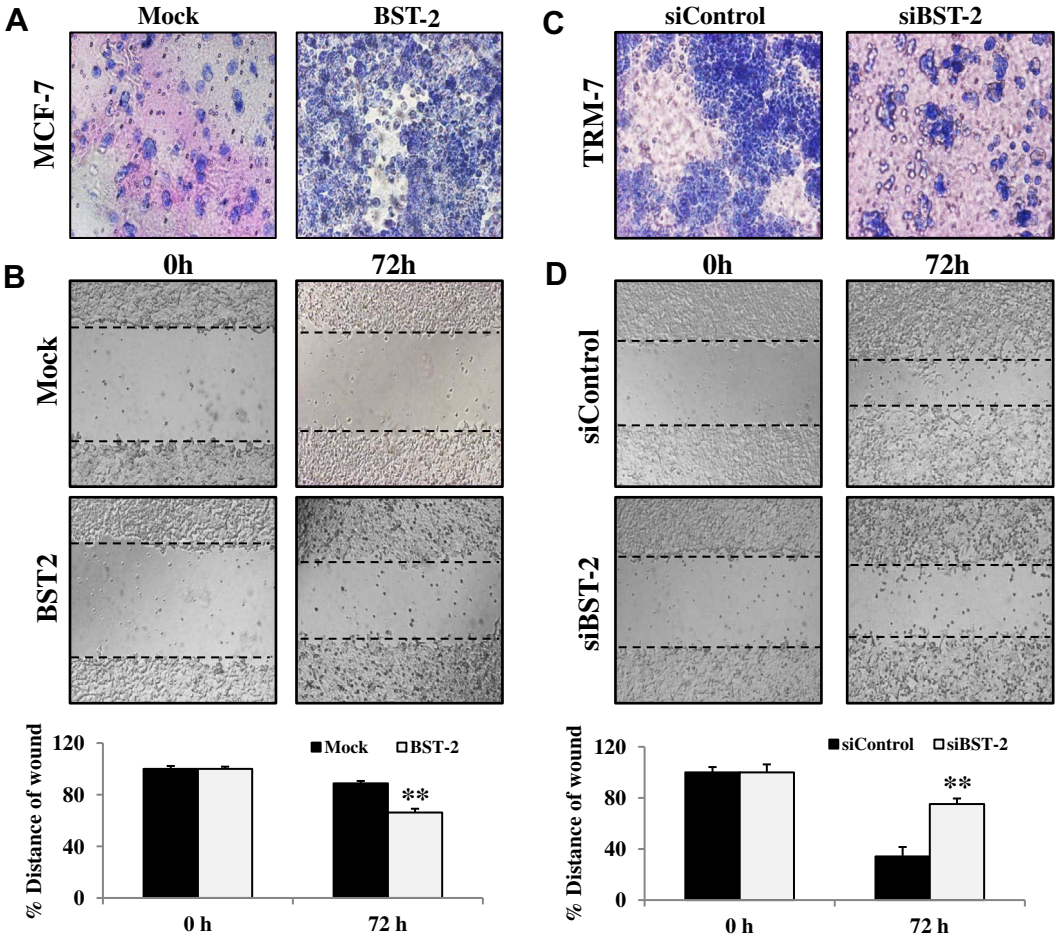

Fig. 2. BST-2 is metastatic factor in breast cancer cells. (A and C) BST-2 construct-transfected MCF-7 cells (A) and siBST-2-transfected TRM-7 cells (C) were seeded onto a Matrigel-coated porous membrane and incubated for 24 h. Invading cells were fixed and subsequently stained using Diff-Quik solution. The original photographs were taken at 10× magnification. (B and D) MCF-7 cells (B) and TRM-7 cells (D) prepared as described above were scratched and incubated for a further 24 h. Wound closure was monitored by measuring wound widths. *Columns*, means of triplicate samples; *bars*, S.D. $^{**}P < 0.01$.

3. Results

3.1. Correlation between metastatic potential and BST-2 expression in TRM-7 cells

We established TRM-7 cells through incubation with tamoxifen for 9 months and described increased tyrosine phosphorylation of STAT3 [8] and invasiveness [1] in these cells. To confirm the metastatic potential of TRM-7 cells, we first performed Matrigel invasion and wound healing assays. We found that TRM-7 cells exhibited greater invasiveness and motility than MCF-7 cells (Fig. 1A and B). To investigate whether BST-2 expression level correlates with invasiveness and motility, we performed Western Blot analysis and found that BST-2 protein levels were increased in TRM-7 cells compared to MCF-7 cells (Fig. 1C). We also confirmed increased mRNA and protein levels of BST-2 in TRM-7 cells by quantitative real-time PCR and immunofluorescence, respectively (Fig. 1D and E). Furthermore, because the possibility that BST-2 could be used as a metastatic novel marker of breast cancer was reported recently [7], we hypothesized that BST-2 may contribute to TRM-7 invasion and migration.

3.2. BST-2 is a metastatic factor in breast cancer cells

To confirm the role of BST-2 in invasiveness and motility in breast cancer cells, we separately overexpressed and knocked down BST-2 in breast cancer cells by transfection with an overexpression vector and siRNA against BST-2, respectively. When MCF-7 cells overexpressed BST-2, their invasiveness and motility were increased compared to parental MCF-7 cells (Fig. 2A and B). By contrast, invasiveness and motility were decreased in TRM-7 cells in which BST-2 expression was silenced by siRNA (Fig. 2C and D). The expression and silencing of BST-2 mRNA were determined by quantitative real-time PCR (Supplementary Fig. 1A and B). These results clearly suggest that BST-2 is an important factor in the invasiveness and motility of breast cancer cells.

3.3. BST-2 induces melanoma nodule growth in a lung metastasis model

We next examined the effect of BST-2 on the growth of metastatic melanoma nodules in mice. BST-2-expressing B16F10 melanoma cells were injected intravenously and then lung specimens were collected on day 14 to investigate the effect of BST-2 on the growth of metastatic melanoma nodules [9–11]. We found that BST-2 expression was much higher in B16F10 cells selected with G418 after BST-2 transfection compared to mock-transfected B16F10 cells (Fig. 3A). The number of metastatic melanoma nodules on day 14 was dramatically increased in the lungs of BST-2-expressing B16F10 cell-injected mice (Fig. 3B). Furthermore, histological assessment by hematoxylin-eosin staining showed infiltrated tumor lesions in the lungs of the BST-2-expressing B16F10 cell-injected mice (Fig. 3C). Consistent with this, we observed increase BST-2 expression in tumor lesions in the lungs of the BST-2-expressing B16F10 cell-injected mice (Fig. 3D). These results clearly indicate that BST-2 plays an essential role in tumor invasion and metastasis.

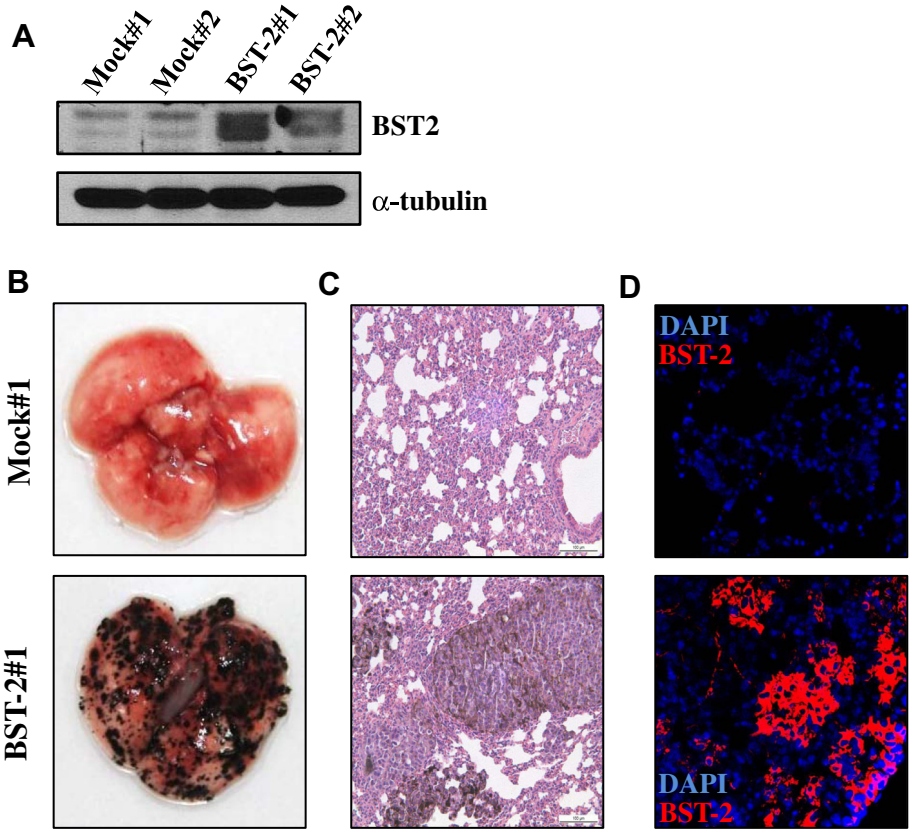

Fig. 3. BST-2 increases metastatic melanoma nodule growth in a lung metastasis model. (A) B16F10 melanoma cells stably expressing BST-2 were generated by selection with G418 for 4 weeks, and BST-2 expression was determined by Western Blotting. (B) B16F10 melanoma cells stably expressing BST-2 were intravenously injected into the tail veins of mice (Mock#1 and BST-2#1). After 14 days, the lungs were collected from the mice for analysis of metastatic colonies ($n = 4$). (C) Hematoxylin-eosin staining of lung metastases. (D) Infiltrated tumor lesions were stained with an anti-BST-2 antibody (red) and nuclei were stained with DAPI (blue). Images (40× magnification) were obtained under a confocal microscope. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

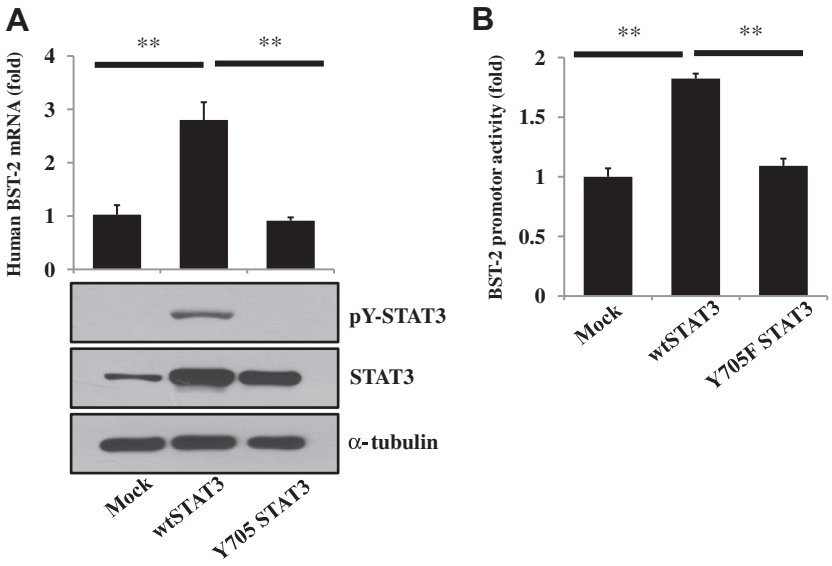

Fig. 4. STAT3 regulates BST-2 expression and promoter activity. (A) MCF-7 cells were transfected with wild-type STAT3 (wtSTAT3) or point-mutated STAT3 (Y705F STAT3). After 24 h, quantitative real-time PCR was performed to measure BST-2 mRNA levels and Western Blotting was performed to measure phosphorylated STAT3 protein levels. (B) MCF-7 cells were co-transfected with a BST-2 reporter construct plus an empty vector (Mock), wtSTAT3, or Y705F STAT3. After 24 h of culture, BST-2-luciferase activity was measured and normalized to that of β-galactosidase. *Columns*, means of triplicate samples; *bars*, S.D. $^{**}P < 0.01$.

3.4. Active STAT3 enhances the expression and transcriptional activity of BST-2

To investigate the mechanism of BST-2 expression associated with breast cancer cell invasion and tumor metastasis, we explored the role of STAT3 in BST-2 expression. The mRNA levels of BST-2 were markedly increased in the presence of wild-type STAT3, but were decreased to basal levels in MCF-7 cells expressing Y705F-mutated STAT3. These data suggest that the expression of BST-2 was dependent on the tyrosine phosphorylation of STAT3 (Fig. 4A). We further examined BST-2 promoter activity and found it to be regulated by STAT3 activation. The transcriptional activity of BST-2 was increased by wild-type STAT3, but was decreased to basal levels in MCF-7 cells expressing Y705F-mutated STAT3 (Fig. 4B). These results suggest that STAT3 can regulate the transcriptional activity of BST-2.

4. Discussion

Tamoxifen resistance is a major challenge in the management of breast cancer patients. Despite an initial response to anti-hormonal therapies, most patients ultimately relapse and present with disease progression [12]. Metastasis in chemoresistant cancer patients is an important clinical problem [13,14]. Therefore, identification and regulation of metastatic biomarkers are important for tamoxifen therapy of resistant breast cancer.

We previously showed that tamoxifen-resistant MCF-7cells exhibited greater invasiveness than parental MCF-7 cells as a result of increased expression of cytokeratin 20 [1]. Furthermore, we reported that STAT3-RANTES autocrine signaling is essential for maintenance of drug resistance and inhibition of programmed cell death [8]. Recently, several researchers reported that BST-2 is highly expressed in many types of cancers [5,7,15] and is a key factor in bone metastasis in breast cancer [7].

In this study, we investigated the role of BST-2 in cancer cell migration and metastasis in tamoxifen-resistant MCF-7 cells. The mRNA and protein levels of BST-2 were significantly increased in tamoxifen-resistant MCF-7 cells compared to parental MCF-7 cells, and high BST-2 expression resulted in increased invasiveness and migration in breast cancer cells. These results indicate the importance of BST-2 in cell invasiveness and migration in breast cancer. Cai and colleagues reported that BST-2 is a potential biomarker associated with bone metastasis in breast cancer [7]. Similar to this report, we also observed that BST-2 increased tumor metastasis in mouse lungs when BST-2-expressing B16F10 melanoma cells were injected intravenously into the tail veins, indicating that BST-2 is an important factor in tumor metastasis.

Phosphorylation of STAT3 at tyrosine 705 was increased in TRM-7 cells [8]. In a variety of human cancers, constitutively activated STAT3 is sufficient to induce tumor metastasis [16–18]. BST-2 has a tandem repeat containing three STAT3-binding motifs between -146 and -126 from the transcription initiation site [6], suggesting that STAT3 may contribute to BST-2 expression [7,19]. In agreement with previous reports [6,7,19], activated STAT3 regulated both the expression and promoter activity of BST-2. Although we could not confirm whether activated STAT3 binds directly to the BST-2 promoter region, STAT3 may contribute to invasiveness, migration, and tumor metastasis by increasing BST-2 expression and promoter activity in breast cancer.

This study provides the first characterization of BST-2 in tamoxifen-resistant breast cancer cells. Although further studies are needed to elucidate the interaction between STAT3 and BST-2, BST-2 is an important factor in invasiveness and tumor metastasis and may be a novel target for new approaches to therapy in patients with metastatic and chemoresistant breast cancer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.043.

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