Exosomal BP1 Protein Could be a Potential Prognostic Biomarker in Breast Cancer

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Abstract

Beta protein 1 (BP1), discovered in our lab, is an isoform protein of the *DLX4* gene that is a member of the homeobox gene family. We have previously shown BP1 mRNA was activated in 80% of invasive ductal breast (IDC) tumors, 100% of estrogen receptor negative (ER^-) tumor tissues and 73% of ER positive (ER^+) tumor tissues. Furthermore, high *BP1* expression levels were associated with tumor cell survival, breast cancer aggressiveness and metastasis. BP1 overexpression was found to stimulate known oncogenes including *BCL2* and *c-Myc*. For the first time we demonstrate that BP1 protein is found in circulating exosomes which are nano-sized membrane-bound vesicles released by various cells and play important roles in intercellular communication. When compared to lower BP1 protein levels found in exosomes from serum of normal Caucasian, exosomes from African American Women (AAW) had high levels of BP1 protein similar to those found in serum exosomes from ER^+ and ER^- stage four metastatic breast cancer Caucasian patients. These results suggest that exosomal BP1 may play a role in the higher mortality rate for AAW with breast cancer. The results also suggest that BP1 protein found in circulating exosomes could serve as a non-invasive prognostic marker for monitoring breast cancer therapies.

Introduction

Breast cancer is the most common cancer in women. Estrogen receptor alpha (ERα) plays a major role in the development and progression of breast cancer. ER-negative breast cancers are usually associated with higher histologic grade, faster proliferation and poorer prognosis. Moreover, the prognosis can vary even at the same stage of cancer [1]. In addition, clinical studies have demonstrated that there are racial disparities among African American women (AAW) and Caucasian women (CW) in breast cancer. Generally, AAW patients have a more aggressive tumor phenotype, higher mortality rate and do not respond well to endocrine therapies [2]. These disparities may be attributed to different status and levels in traditionally "undruggable" molecules, such as transcription factors, that are required for the oncogenic phenotype. Those molecules could be novel biomarkers for predicting the likelihood of recurrence and estimating the probability of developing breast cancer in the women with different hormone receptor status.

Homeotic proteins, which contain a highly conserved homeodomain (HD), are transcription factors that function as master regulators in embryogenesis and organogenesis. The HD is composed of 60 amino acids, forming three alpha helices [3, 4]. Homeotic proteins are involved in malignancies, including breast cancer [5, 6, 7]. Overexpression of HOXA1, for example, resulted in Bcl-2 up-regulation, increased anchorage-independent growth and tumor formation in mice [8]. HOXB7 overexpression in SkBr3 breast cancer cells increased their growth rate, serum-independent growth and anchorage independence, as well as their ability to form tumors in mice [9, 10]. In contrast, methylation of the promoter of HOXA5 led to decreased expression of the important tumor suppressor gene p53 [11].

Beta protein 1 (BP1), discovered in our lab, is an isoform protein of the *DLX4* gene that is a member of the homeobox gene family of transcription factors. Previous work showed that: (i) There is a strong correlation between *BP1* expression and the estrogen receptor status. BP1 mRNA was activated in 80% of invasive ductal breast (IDC) tumors, where 100% of estrogen receptor (ER) negative tumor tissues and 73% of ER positive tumor tissues were BP1 positive [12]. (ii) BP1 expression may also be associated with ethnic differences. 89% of the tumors of African American women expressed BP1 while 57% of Caucasian women showed BP1 positivity [12]. (iii) High BP1 levels were associated with tumor cell survival, breast cancer progression and metastasis. In 0% of normal adult breast tissue, 21% of ductal hyperplasia, 46% of ductal carcinoma in situ (DCIS), 81% invasive ductal carcinoma and 100% of inflammatory breast cancers, BP1was positive [13]. Further study in ER-negative Hs578T cells confirmed that BP1 overexpression correlates with increased cell proliferation and enhances the in vitro invasive activity [14]. Experiments in a mouse model showed that overexpression of BP1 also could increase tumor size and lead to estrogen-independent tumor formation [15]. (iv) BP1 overexpression was found to stimulate known oncogenes, including *BCL2* and *c-Myc*. Thus, high BP1 expression can lead to increased proliferation and increased differentiation [16, 17].

Exosomes are small bilayered extracellular vesicles (EVs) with a characteristic diameter of 40-100 nm measured by electron microscopy [18]. Exosomes contain large amounts of proteins, lipids and nucleic acids, which suggests their complexity. Many studies have demonstrated that cancer released exosomes play a crucial role in oncogenesis and progression [19]. In recent years, exosomal proteins have shown their unique characteristics compared to traditional biomarkers for carcinoma diagnosis and prognosis. First, exosomal proteins are more sensitive than the proteins directly detected in liquid biopsy [20, 21]. Second, exosomal proteins are easily attainable for clinical detection, because exosomes are widely found in various body fluids [22]. Moreover, the lipid bilayer structure prevents exosomal proteins from being degraded by enzymes, which makes them very stable [23]. The above features highlight the feasibility and advantages of developing exosomal proteins as biomarkers for early detection, monitoring and prognosis prediction for cancer.

Therefore, the aim of this study was to evaluate the tumorigenic effects of secreted BP1 protein from breast cancer cells, verify that BP1 protein mainly released in an exosome-associated form, and analyze exosomal BP1 protein

levels in clinical serum samples. Our results showed that exosomal BP1 protein could provide a novel biomarker for prognosis of breast cancer.

Materials and methods

Cell Culture
Breast adenocarcinoma cell lines MCF-7 and T47D were maintained in RPMI1640 media (Gibco), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S), and MDA-MB-231 were maintained in DMEM media (Gibco), supplemented with 10% FBS and 1% P/S. MCF10A and H16N2 cells were maintained in Mammary Epithelial Cell Media (Cambrex), supplemented with 0.4% bovine pituitary extract, 0.1% human epidermal growth factor, 0.1% insulin, 0.1% hydrocotisone, and 0.1% gentamycin (Cambrex). H16N2 cells were the kind gift of Dr. Vimla Band. The cells were cultured with 5% CO_2 at 37 ℃.

Conditioned Media (CM)
Cells were grown for 3 days, and CM was concentrated 200-fold, using YM-10 (Millipore) or YM-70 concentrators, depending on the number of cells grown. 200x CM was diluted 20-fold in media to obtain 10x CM.

Recombinant BP1 protein (rBP1 protein)
BP1 cDNA was cloned in a pET28a vector containing a hexa-histidine tag and transformed into BL21α *E. coli* cells. Cells were lysed after induction with 1 mM IPTG, and the soluble fraction was prepared for column purification. rBP1 protein was purified on a nickel column. Western blot analysis using anti-BP1 protein antibody, a gift of Rockland Immunochemicals (600-403-MS7), or an anti-histidine antibody confirmed BP1 protein purification.

Fluorescent immunohistochemistry
Cells were fixed in paraformaldehyde (Sigma) for 20 minutes and permeabilized in 0.2 % Triton X-100 (Sigma). After blocking with goat serum (Zymed), cells were probed with primary and secondary antibodies diluted as described (Table 1). Confocal microscopy was performed on a BioRad MRC 1024 using a 15mW Krypton/Argon mixed-gas laser.

Western Analysis
Western blot was performed as described in our previous study [16] using antibodies as shown in Table 1.

Measurement of Cell Viability
Cell viability was measured by the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyltetrazolium bromide dye conversion assay). 2,000 cells were grown, treated, and incubated with 5 mg/ml MTT for 4 hrs. Formazan was released by DMSO (Sigma) from living cells and the absorbance was read at 570nm. Data was normalized against the mean value of each control group, which was set to 1 on day 0.

Quantitative Real-time RT-PCR
Total RNA was extracted from cells with Trizol (Invitrogen). 1µg of RNA was reverse transcribed for 10 min at 25° C, 30 min at 50 °C, and 5 min at 85° C using Superscript III kit (Invitrogen). cDNA was subject to real-time PCR (ABI 7000) with SYBR green (ABI) as follows: 95 C° for 10 min (1 cycle), 95 C° for 15 sec (40 cycles), and 60 C° for 1 min (1 cycle). BP1 and Bcl-2 primers were described in previous study [16]. Twist and Met primers were purchased from SuperArray. Results were normalized against 18S rRNA.

Exosome Isolation
Exosomes were isolated by Exosome Precipitation Solution (ExoQuick, System Biosciences) and quantified using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). The absorbance was read at 562nm in 96-well plates

using an ELISA reader (BioTek). The exosomes isolation was performed for all the cell lines and serum samples according to the protocol described in the manufacturer's instructions. Finally, the exosome pellets were resuspended with sterile 1X PBS and stored at -20°C. Exosomes were processed for visualization by Transmission Electron Microscope (TEM) as previously described [24].

Clinical Serum Samples

Human serum from women with metastatic breast cancer and normal control serum samples were purchased from Capitol Biosciences. According to the datasheet and protocol from Capitol Biosciences, all these serum samples were collected from women after fasting. The metastatic breast cancer samples were taken from Caucasian patients before treatment.

In-capillary immunoassay

An in-capillary immunoassay platform, Simple Western (Protein Simple, San Jose CA), was used according to the procedure described in an earlier study [25].

Statistical analyses

All results were expressed as the mean ± SD form. Means were calculated from at least three independent experiments. Two-tailed unpaired Student's t-test was used for the comparison of different groups. Analyses were performed using GraphPad Prism 7.0 (GraphPad Software). A value of $P < 0.05$ was considered to be statistically significant. In this study, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 1. Antibodies for Western blot analysis (WB), immunofluorescence (IF), and immunoprecipitation (IP)

Antibody	Use	Concentration
Anti-human BP1 rabbit polyclonal IgG (Rockland Immunochemicals, Inc.)	WB	1:1000
	IF	
	IP	10 µg per 500 µl of 200x CM
Anti-histone H3 mouse monoclonal IgG (Upstate)	WB	1:2000
Anti-rabbit IgG AlexaFluor 488 (Invitrogen)	IF	1:500
Anti-mouse IgG AlexaFluor 568 (Invitrogen)	IF	1:500

Results

BP1 protein is secreted by breast cancer cells but not by normal breast epithelial cells

Conditioned media (CM) from four breast cancer cell lines, MCF-7, T47D, MDA-MB-231 and MCF-7 O2 cells (MCF-7 overexpressing *BP1*), as well as MCF10A and H16N2 normal breast epithelial cells, was collected and concentrated; 10x CM was analyzed by Western blot with anti-BP1 antibody (Figure. 1A). The characteristic BP1 protein band at 36kDa was observed in cell extracts (CE) from MCF-7, T47D, MDA-MB-231 and MCF-7 O2 breast cancer cells (lanes 1, 3, 5 and 13), and also in the 10x CM from these cells (lanes 2, 4, 6 and 14). This band was not seen in 10x CM from MCF10A or H16N2 normal cells (lanes 8, 10 and 12), although the BP1 protein band appeared in CE from both cell lines (lanes 7, 9 and 11). To verify that BP1 protein was not present in CM due to release by dead cells, 10x CM from MCF-7, T47D, MDA-MB-231 and MCF-7 O2 cells was analyzed by Western blot with anti-histone H3 monoclonal Ab (Figure. 1B). The histone H3 protein is 16kDa and was detected in CE from MCF-7, T47D, MDA-MB-231 and MCF-7 O2 cells (lanes 1 through 4), but not in CM (lanes 5 through 8), indicating there was not significant cell lysis and allowing us to conclude that BP1 protein in the media was not due to dead cells.

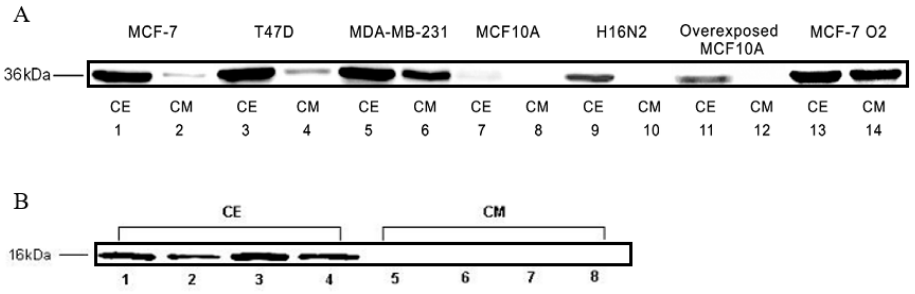

Figure 1. BP1 is secreted by breast cancer cells but not normal epithelial cells. Cells were grown and CM was isolated as described in Materials and Methods. 10 µg of cell extract (CE) and 30 µl of 10x CM were analyzed by Western blot. (A) Lanes 1, 3, 5, 7, 9 and 13 contain CE from MCF-7, T47D, MDA-MB-231, MCF10A, H16N2 and MCF-7 O2 cells, respectively. Lanes 2, 4, 6, 8, 10 and 14 contain 1X CM from these cells. BP1 protein, a 36 kDa band, is marked. MCF-7 O2 cells (lanes 13 and 14) were analyzed on a separate gel and merged with the image from the other cell lines. A longer exposure of MCF10A CE and CM, corresponding to lanes 7 and 8, is shown in lanes 11 and 12. (B) The histone H3 protein which is 16kDa was detected in CE from MCF-7, T47D, MDA-MB-231 and MCF-7 O2 breast cancer cells (lanes 1 through 4), but not in CM from these cells (lanes 5 through 8).

Secreted BP1 protein is internalized by cells.

To confirm the internalization of BP1 protein secreted from breast cancer cells, a labelling assay was performed and the result was visualized by confocal microscopy. Because of the relatively high secretion of BP1 protein by MCF-7 cell lines overexpressing *BP1* (MCF-7 O2 cells), they were used as donors in co-culture experiments with MCF-10A normal breast epithelial cells, which only have very low levels of BP1 protein inside, as recipients. BP1 protein levels clearly increased in both the cytoplasm and nucleus of MCF-10A cells co-cultured with MCF-7 O2 cells, compared with cells co-cultured with media alone, demonstrating that secreted BP1 protein from breast cancer cells was internalized by MCF-10A recipient cells and transported into the nucleus (Figure 2A). This was verified using a recombinant, histidine tagged recombinant BP1 protein (rBP1). rBP1 protein was added to MCF-10A or MCF-7 cells at a concentration of 1 ng/ul and detected by confocal microscopy using anti-his antibody (Figure 2B&C). Clearly, rBP1 protein was internalized by both cell types. To determine the relative numbers of MCF-10A normal cells that were converted to BP1 protein positivity, MCF-10A cells were immunostained after three days in similar co-culture experiments (Figure 2D). Cells were scored as weakly staining (arrow heads) or intensely staining (arrows). Approximately 8% (72/865) of control cells were weakly BP1 protein positive compared with 80% (275/343) of treated cells; among treated cells, about 10% were intensely staining. These results showed that secreted BP1 protein was internalized by MCF-10A cells, transported to their nuclei and eventually converted them to highly enriched BP1 protein positive cells.

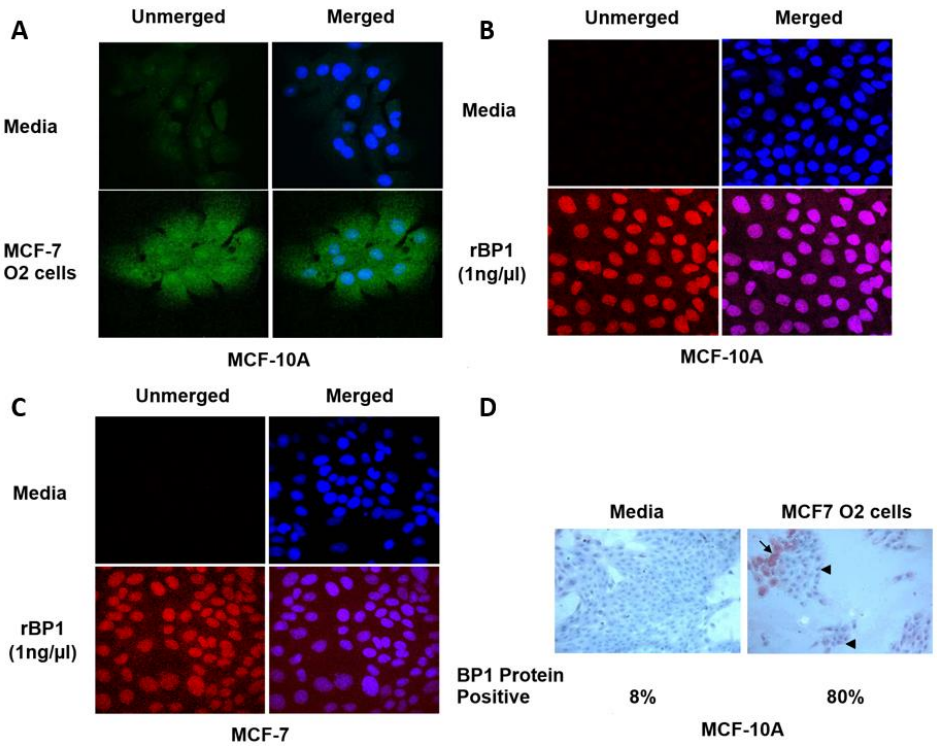

Figure 2. Subcellular localization and relative concentration of BP1 protein. Cells were grown and subjected to fluorescent immunohistochemistry. (A) BP1 protein is secreted by MCF-7 O2 cells and internalized by MCF-10A cells. The donor in trans-well plates is indicated on the left, either a media control or MCF-7 O2 cells. The pore size allowed BP1 protein but not cells to traverse the filter separating the chambers. MCF-10A cells were recovered from the bottom chamber after three days of growth. Labeled BP1 protein is identified by green immunofluorescence, whereas the nucleus, stained with TOTO reagent, is blue; (B & C) MCF-10A & MCF-7 cells were grown in rBP1 protein or media alone, and internalized the recombinant protein. Labeled histidine tagged rBP1 protein is identified by red immunofluorescence, whereas the nucleus is blue; (D) Trans-well plates were used to determine the frequency of conversion of MCF-10A cells to highly enriched BP1 protein positive cells. BP1 protein positive cells were counted by two independent observers.

Secreted BP1 protein exhibits mitogenic activity, which is related to cancer metastasis.

To test the effects of secreted BP1 protein on cell viability, MCF-7, T47D, MDA-MB-231 breast cancer cells and MCF-10A normal epithelial cells were grown for 7 days in unsupplemented media or media containing a final concentration of 10x Cell Media (diluted from 200x CM) derived from MCF-7 O2 cells, T47D cells and MCF-10A, respectively. As a control, BP1 protein was cleared by two rounds of immunoprecipitation resulting in Depleted Media (DM, Figure 3), resulting in a loss of ability to stimulate proliferation. By day seven, the proliferation of cells grown in 10x CM from MCF-7 O2 cells was uniformly higher than cells grown in unsupplemented media. Remarkably, the viability of MCF-10A cells was also enhanced by treatment with 10x CM. DM was not mitogenic for MCF-7, T47D, MDA-MB-231 or MCF-10A cells. In addition, growth was statistically lower in Depleted Media (DM) for all cell lines except MCF-10A (Figure 4A). The same cell lines were grown in 10x CM derived from T47D cells, which also secrete BP1 protein; results were parallel to those using 10x CM from MCF-7 O2 cells (Figure 4B). Moreover, there was no mitogenic effect when any of the four cell lines was grown in the presence of 10x CM from MCF-10A cells, which lack secreted BP1 protein (Figure 4C). Mitogenic effects of BP1 protein alone were assessed by growth of MCF-7 and MCF-10A cells in media supplemented with 1 ng/ul recombinant BP1 protein (rBP1 protein). Stimulation was 1.8-fold in MCF-7 cells and 2.2-fold in MCF-10A cells by day 5 of growth (Figure 4D). These data and the results showing that CM depleted of BP1 protein did not stimulate growth suggest that the presence of BP1 protein in the media is necessary and sufficient for the mitogenic effects of secreted BP1 protein.

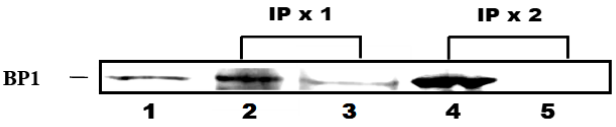

Figure 3. BP1 protein in conditioned media was depleted by immunoprecipitation. 200x CM from MCF-7 O2 cells was immunoprecipitated with 10µg of anti-BP1 antibody. Lane 1 is positive control. BP1 protein was successively depleted in the supernatant of CM after one round or two rounds of immunoprecipitation (Lanes 3 and 5, respectively), while increased BP1 protein was observed in the second immunoprecipitate (Lane 4) compared with the first one (Lane 2).

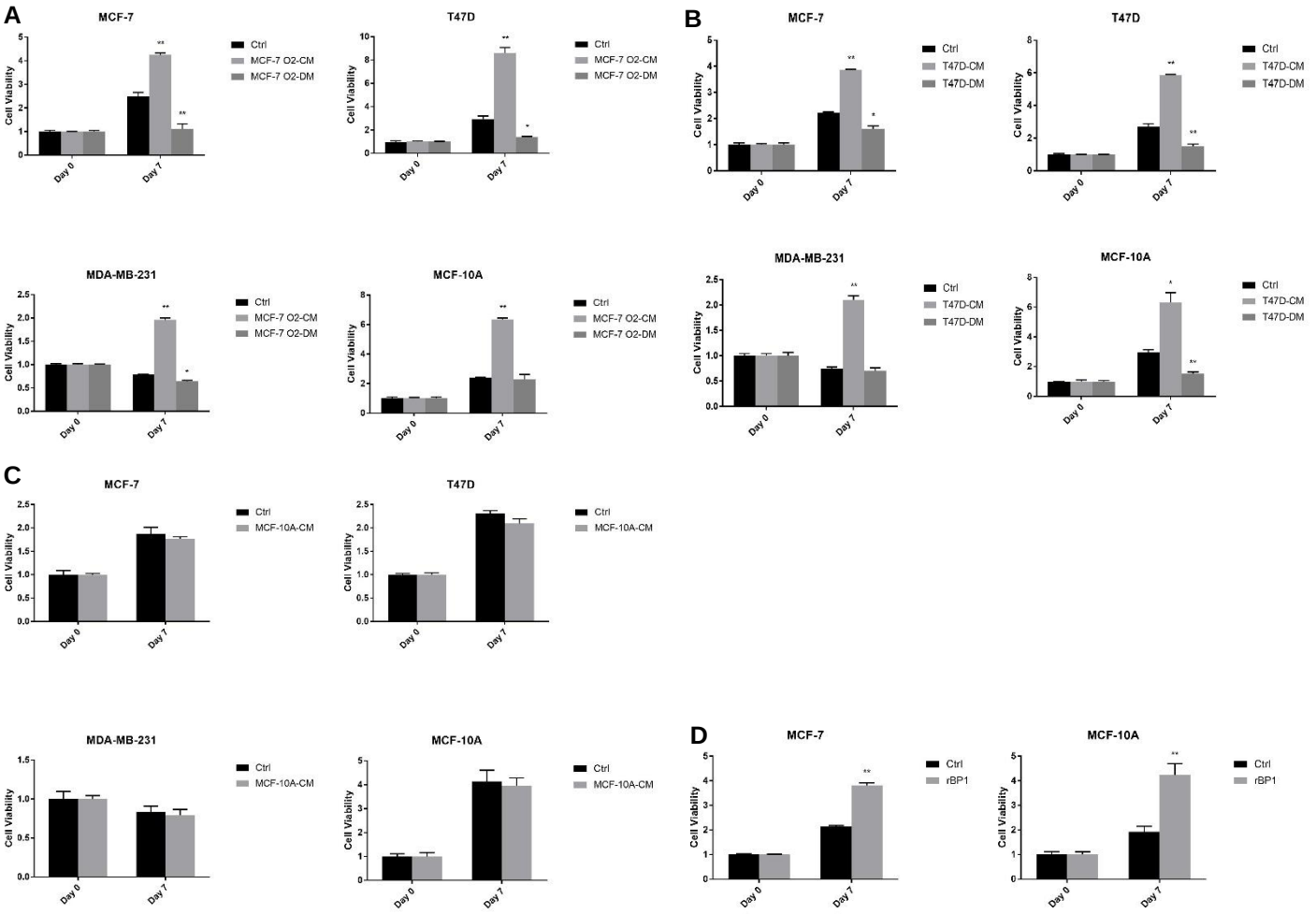

Figure 4. Secreted BP1 protein is mitogenic. Cells were grown, and media was collected after three days, concentrated 200-fold, and diluted in media to a final concentration of 10x. Depleted medium (DM) was prepared from CM by two rounds of immunoprecipitation to remove detectable BP1 protein. Two or more preparations of CM and DM were tested. Cell growth was measured independently at least twice in triplicate, using the MTT assay. Values are normalized to the mean value of each control group in Day 0. (A) Breast cell lines MCF-7, T47D, MDA-MB-231 and normal control MCF-10A were grown in media containing 10x CM from MCF-7 O2 cells or in DM for 7 days. (B) Breast cancer cell lines MCF-7, T47D, and MDA-MB-231 or normal breast cell MCF-10A were grown in media containing 10x CM from T47D cells or in DM for 7 days. (C) Breast cell lines MCF-7, T47D, MDA-MB-231 and normal control MCF-10A were grown in media containing 10x CM from MCF-10A for 7 days. (D) The mitogenic effect of 1 ng/µl rBP1 protein was tested on MCF-7 cells and MCF-10A cells. In each panel, growth was analyzed by comparing the control (complete media) with 10x CM or comparing the control with 10x DM or comparing the control with 1 ng/µl rBP1 group. Bars represent mean cell viability ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Secreted BP1 protein regulates oncogene expression

To examine potential molecular targets of internalized BP1 protein, several oncogenes up-regulated by BP1 protein overexpression in MCF-7 O2 cells were tested, including Bcl-2 and Met. High level Bcl-2 expression results in increased migration, invasion and angiogenesis, while high Met expression is associated with tumor growth, invasiveness and metastasis, making both genes important targets of BP1. Auto-stimulation of BP1 mRNA was also assessed. Table 2A shows the results of gene expression analysis of recipient cells in different co-culture experiments in which MCF-7 O2 cells were the donor. In the chamber experiments in A, after 3 days of growth in which MCF-7 O2 cells were the donor and MCF10A cells were the recipient, Met was up-regulated 1.6 fold ($P<0.01$), and BP1 mRNA was up-regulated 2.8-fold ($P<0.001$). There was no change in expression of Bcl-2. In contrast, when MCF-7 cells were recipients and MCF-7 O2 cells were donors, Bcl-2 and Met were up-regulated, but not BP1 ($P<0.01$. $P<0.05$, $P<0.01$, respectively). We also looked at changes in mRNA expression of the same genes after growth of MCF-7 cells in 10x CM (Table 1B). Here, we observed up-regulation of Bcl-2 ($P<0.001$) and Met ($P<0.01$) after seven days, but again there was no change in BP1 mRNA levels. Finally, MCF-7 cells were grown in 1 ng/ml of rBP1 protein as positive control, where again Bcl-2 and Met were up-regulated ($P<0.001$, Table 1C). Thus, the same oncogenes were stimulated in MCF-7 cells whether they were grown in co-culture with MCF-7 O2 cells, in 10x CM derived from MCF-7 O2 cells, or in the presence of rBP1 protein.

Table 2. Analysis of gene expression in the presence of secreted BP1 protein

	(A) CHAMBER		(B)10X CM	(C) rBP1
GENE	**MCF 10A**	**MCF-7**	**MCF -7**	**MCF-7**
Bcl-2	N.C.	1.4 ± 0.07	1.8 ± 0.06	2.7 ± 0.08
Met	1.6 ± 0.09	1.4 ± 0.08	1.6 ± 0.08	2.5 ± 0.05
BP1	2.8 ± 0.03	N.C.	N.C.	N.C.

Secreted BP1 protein is released predominantly in an exosome-associated form from breast cancer cells.

Exosomes were isolated from MCF-7, T-47D and MDA-MB-231 breast cancer cell lines using Exosome Precipitation Solution (ExoQuick, System Biosciences) which is suggested to be a more efficient method for isolating exosomes [26]. Electron microscopy of purified exosomes showed the characteristic cup shaped vesicles in the correct size range (30-100nm) (Figure 5). Western blot revealed exosomes-associated BP1 protein from MDA-MB-231 cells (Figure 6). BP1 protein was observed in only the exosomal fraction and cell extract (lane 4 and 5, respectively), but not in the supernatant or washes, strongly supporting the idea that BP1 protein is mainly secreted in an exosome-associated form.

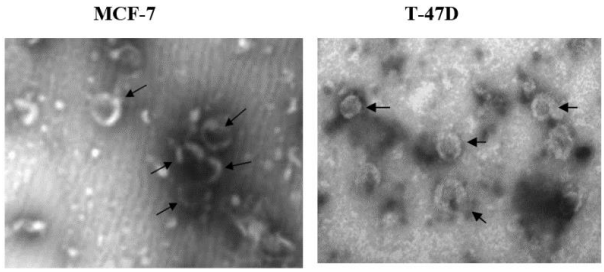

Figure 5. Electron microscopy of exosomes from MCF-7 and T-47D cells. Arrows indicate exosomes, which exhibit the expected characteristic cup-shaped morphology.

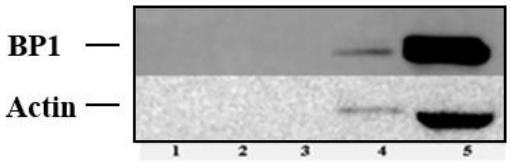

Figure 6. BP1 protein from exosomes derived from MDA-MB-231 breast cancer cells. Lane 1, conditioned media before concentration (very low amount of exosomal protein expected); Lane 2, supernatant after centrifugation during exosome isolation; Lane 3, 1X PBS after washing of the exosomal pellet; Lane 4, exosome pellets; Lane 5, cellular extract from the same cells. The bands in the lower panel demonstrated the presence of actin in both the exosomal fraction as well as in the cellular extract, as expected.

Exosomal BP1 protein levels were significantly higher in breast cancer serum samples compared to normal controls.

BP1 protein was detected by Western blot analysis in exosomes isolated from human serum samples purchased from Capital Biosciences, where Metastatic Breast Cancer (MBC) samples presented stronger bands than Normal Human Serum (NHS) groups (Figure 7). In order to quantitate exosomal BP1 protein in the serum more precisely, Simple Western, which is more sensitive and reproducible than Western blot, was performed. First, rBP1 was employed to build a standard curve (Figure 8). The results of Simple Western assays suggested that exosomal BP1 concentrations (ng/ul) in the serum of metastatic breast cancer Caucasian patients (N=30) were significantly higher than the normal controls (excluded AA samples, N=20) (**P < 0.01) (Figure 9A). There is no significant difference between ER negative patients and ER positive patients. In the normal control groups, African American samples tend to have higher exosomal BP1 concentrations in the serum. Furthermore, according to the exosomal protein levels in the serum of breast cancer patients and control groups, BP1 protein levels in 1 ug exosomal protein were also analyzed. In general, MBC serum groups (N=30) have higher proportion of BP1 protein in the exosomal protein compared to normal controls (excluded AA samples, N=20) (**P < 0.01) (Figure 9B). Whole exosomal proteins in the African American women tend to have significant higher percentage of BP1 protein compared to Caucasian women (*P < 0.05).

Figure 7. Presence of BP1 protein in exosomes isolated from serum in Western blot. BP1 protein bands were observed in Western blot analysis, which indicated that BP1 protein existed in the exosomes isolated from serum of women with metastatic breast cancer (MBC) and Normal Human Serum (NHS). rBP1 was employed as standard and positive control.

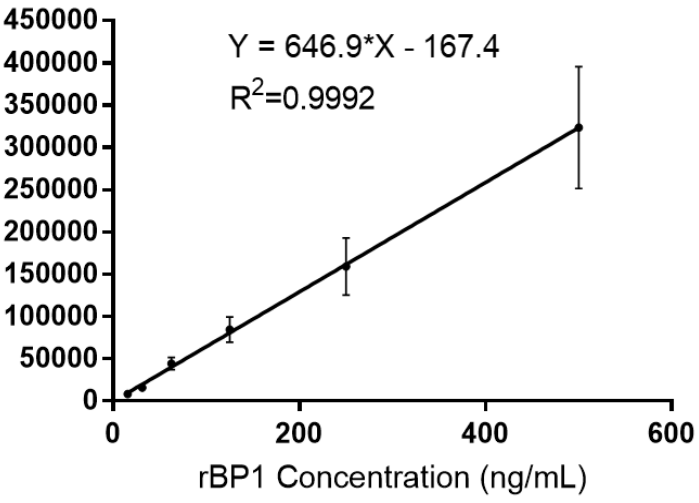

Figure 8. Standard curve generated from recombinant BP1 protein in a serial dilution using Simple Western. Two-fold dilutions were made from 500ng/ml to 15.625ng/ml.

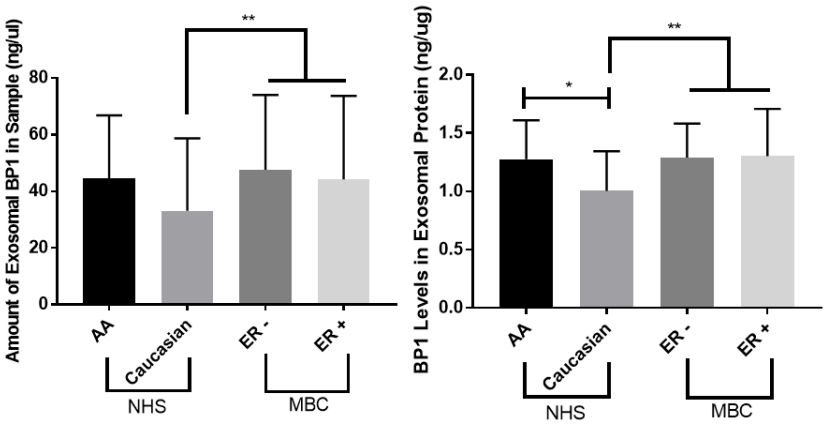

Figure 9. Simple Western assay was performed to quantitate exosomal BP 1 protein in the serum from 60 samples. 30 Caucasian Metastatic Breast Cancer patients (MBC), including 15 ER^+ and 15 ER^- patients. 30 Normal Human Serum (NHS) from women, including 10 African American (AA) samples and 20 Caucasian Samples. (A) The concentration of exosomal BP1 protein in the serum; (B) The proportion of BP1 protein in the exosomal protein in each sample. ER=Estrogen receptor, *P < 0.05, **P < 0.01.

Discussion

We found that the homeotic protein BP1 is secreted by three breast cancer cell lines, MCF-7, T47D and MDA-MB-231, a derivative of MCF-7 cells overexpressing BP1 called MCF-7 O2 cells, but not by normal breast (MCF10A and H16N2) cells. It has been hypothesized that homeoprotein secretion is a highly regulated process, which our data support in the case of breast cancer cells. The basis of this regulation is not yet known but might involve secretion via vesicles according to our results. Secretion of free homeotic proteins also can occur and is known to be dependent on a highly conserved short amino acid sequence in the HD. The BP1 protein amino acid sequence shows a 60% match to this sequence (Table 3). Future experiments will be directed toward understanding this regulation. We speculate that BP1 protein may also be secreted by other malignant cells that are BP1 positive, such as acute myeloid leukemia cells [27]. To our knowledge, this is the first demonstration that malignant cells can secrete a homeoprotein/transcription factor in a exosomes-associated form.

Internalization of the HD of Ant and other homeotic proteins requires 16 amino acids in the third helix of the HD [28]; BP1 protein shows 75% amino acid homology to this motif (Table 3). Internalization of homeotic proteins or purified homeodomains has been observed for all HD tested, probably due to the high conservation of the third helix [28]. Tryptophan at position 48 (W48) is a crucial amino acid for internalization. BP1 has conserved this important amino acid, which is the sixth amino acid in the internalization signal (Table 2). One model of internalization is that peptide binding to the cell surface occurs through charge interactions followed by membrane destabilization after insertion of W48 in the membrane bilayer. Inverted micelles might form, allowing proteins to translocate across the membrane and be released into the cytoplasm [28]. Due to the high conservation of protein sequences among homeotic proteins, we hypothesize that other homeotic proteins may be secreted by malignant cells and internalized by both malignant and normal epithelial cells.

The biological impact of endogenous homeoprotein secretion and internalization has not previously been determined. We tested the mitogenic capability of secreted BP1 protein using several approaches. (i) Breast cancer cells were grown in un-supplemented media, which contains secreted BP1 protein, and in media supplemented with CM to a final concentration of 10x, thus increasing the concentration of secreted BP1 protein. All three cell lines grew significantly better in the presence of higher levels of BP1 protein. Unexpectedly, the growth of MCF10A cells was enhanced in 10x CM, which implies there may be paracrine effects of BP1 protein secreted by tumor cells *in vivo*, with biological effects. The growth of MCF-7, T47D, MDA-MB-231 and MCF10A cells was not stimulated by 10x CM from MCF10A cells, which do not secrete BP1 protein. (ii) Cells were grown in 10x CM depleted of BP1 protein (DM). Cells grown in DM did not show growth stimulation compared with cells grown in 10x CM, a strong indication that BP1 protein is required for optimal growth of breast cancer cells. (iii) 10x CM from T47D cells was also mitogenic for breast cancer and normal breast cells, demonstrating that MCF-

7 cells are not unique in secreting active BP1 protein. (iv) Recombinant BP1 protein alone was mitogenic for not only breast cancer, but normal breast cells, supporting the idea that BP1 protein is necessary and sufficient for the mitogenic effects we observed in breast cancer.

These experiments show that secreted BP1 protein has autocrine activity since growth stimulation was seen in cells grown in CM (but not in DM) from the same cells, e.g., CM from MCF-7 O2 cells was mitogenic for parental MCF-7 cells, as well as for O2 cells themselves; CM from T47D cells stimulated growth of T47D cells. Paracrine activity was seen upon stimulation of normal breast and prostate epithelial cells with CM. Our clinical data are consistent with the notion that BP1 protein may have paracrine activity *in vivo* - we have observed that pathologically normal breast cells and prostate cells near BP1 positive tumor cells are more frequently BP1 immunopositive than normal cells at a distance from the tumor [29]. We hypothesize that other homeotic proteins expressed in breast cancer cells may also be secreted, with autocrine and paracrine functions.

Molecular events in recipient cells following the addition of secreted BP1 protein or rBP1 protein were assessed by measuring expression of selected genes already determined to be regulated by BP1 protein in breast cancer cells, including Bcl-2 and Met. Bothe these oncogenes are up-regulated in MCF-7 cells using the chamber assay, growth in 10x CM or growth in rBP1 protein. We predict that activation of these or other targets of BP1 may occur in tumor cells that express high BP1 levels. These results are consistent with our observation that increasing BP1 protein levels in MCF-7 cells, results in increased aggressiveness [15]. Importantly, growth of MCF10A cells in 10x CM stimulated expression of not only Met, but of BP1 itself. If this occurs *in vivo* with normal epithelial cells located near BP1 positive tumor cells, an altered program of gene expression may be initiated in the normal cells. This will be an important area to explore in the future.

In addition, our research showed secreted BP1 protein predominantly existed in the exosomes isolated from breast cancer cell lines. In this regard, the exosomes isolated from women's serum were run a Western blot to screen for exosomal BP1. An in-capillary immunoassay platform (Simple Western) was used to evaluate the exosomal BP1 protein levels. Exosomes' concentration is reportedly increased in the blood of patients with ovarian and pancreatic cancer when compared with normal controls [30]. Moreover, the cargo of tumor-derived exosomes can change according to the patient's health status [31]. In order to have a more precisely comparison, BP1 protein levels in 1 ug exosomal protein were also analyzed. It was found that the exosomal BP1 concentrations and the BP1 percentage levels in the whole exosomal protein were both significantly higher in the serum from breast cancer patients. Moreover, exosomal proteins in the African American women tend to have significant higher percentage of BP1 protein compared to Caucasian women. This may indicate an unfavorable role of exosomal BP1 protein in breast tumor development in African American women. Thus, exosomal BP1 protein may be a good prognostic biomarker or therapeutic target in breast cancer.

This is the first report of BP1 protein in exosomes purified from serum of women with breast cancer and normal healthy women. In addition, the evaluation of this important family of transcription factors, *DLX4*, in the treatment and understanding of breast cancer is also quite novel. In further studies, it may be desirable to develop new model systems to unravel the mechanism for the functions of exosomal BP1 protein in tumorigenesis. Furthermore, a more thorough understanding of the relationship between exosomal BP1 protein and racial disparities in breast cancer may lead to the confirmation of the role of exosomal BP1 protein as a potential breast cancer prognostic marker and to the development of potential therapeutics of breast carcinoma.

Table 3. Conservation of secretion and internalization signals

	Secretion Signal										Match
Ant/En2	**A**	**Q**	**E**	**L**	**G**	**L**	**N**	**E**	**S**	**Q**	
BP1		X					T	Q	T		60%
HOXA1		A	S		Q				T		60%
HOXB7		H	T		C		T		R		50%
HOXA5		H	A		C		S		R		50%

	Internalization Signal																Match
Ant/En2	**R**	**Q**	**I**	**K**	**I**	**W**	**F**	**Q**	**N**	**R**	**R**	**M**	**K**	**W**	**K**	**K**	
BP1	T									K		S		Y			75%
HOXA1	T		V											Q			81%
HOXB7																	100%
HOXA5																	100%

Table 3. The canonical secretion and internalization amino acid signals from Ant/En2 are shown in bold. For the genes being tested, only the presence of a different amino acid is shown. An X means an amino acid is missing from the BP1 protein sequence. For HOXC6, there is overlap at RQ between the putative secretion signal and the putative internalization signal.

Conflict of Interest

The authors declare no conflict of interest.

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