Cancer-Associated Fibroblasts Release Exosomal CBFB That Dictate an Aggressive Bone Metastasis Phenotype in Breast Cancer

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Research

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Abstract

Background

Breast cancer up to date remains one of the most prevalent female malignancies in the world. Better prognostic and therapeutic biomarkers are urgently required for these patients. Circulating exosomes are shown to participate in tumorigenesis including distance metastasis and of prognostic/therapeutic potential.

Methods

Sera from control health, primary breast cancer, and bone metastatic breast cancer patients were collected. The Exosome were isolated from collected sera and culture medium from previously steps, and a standard procedure was performed. We utilized MDA-MB-436-derived xenograft mouse model to demonstrate that silencing CBFB (core binding factor subunit β) significantly reduced bone-metastasis in association with reduced expression of OPN, IL-6, Runx2 and OPN as well as reduced exosomes containing CBFB.

Results

We found that circulating exosomes (Exos), from bone metastatic patients with breast cancer, were enriched with CBFB. Fibroblasts co-cultured with Exos showed increased α-SMA, vimentin expression and increased secretion of IL-6 and OPN; non-metastatic breast cancer cells co-cultured with Exos exhibited increased markers including vimentin, snail 1, CXCR4 and Runx2. Subsequent analysis revealed that these Exos were enriched with bone metastasis associated marker CBFB. Gene-silencing experiments metastatic MDA-MB-436 and MDA-MB-157 cells, demonstrated that CBFB significantly reduced metastatic potential, reflected by the suppression of vimentin, CXCR4, snail 1 and Runx2, CD44 and OPN. In contrary, CBFB-overexpression resulted in the increased metastasis associated genes in non-metastatic T47D and MCF7 cells. The CBFB-enriched exosomes derived from MDA-MB-436 enhanced metastatic phenotypes of low metastatic potential breast cancer cell lines.

Conclusion

We demonstrated the essential roles of CBFB in the promotion of bone metastasis in breast cancer cells. The suppression of CBFB led to the decreased tumor burden and bone metastasis in association with decreased markers of bone metastasis including CXCR4, Snail, CD44, OPN, Runx2 and IL-6.

Background

Breast cancer is one of the most common cancer types in the female population. It was estimated that breast cancer represents approximately 30% of all new cancer diagnoses in women in 2016 in the US [1, 2]. Despite the advance in the development of therapeutic interventions; the treatment options for distant metastatic cases remain limited and efficacy limited. In addition; there is a lack of reliable
prognostic/therapeutic biomarkers. Thus, in order to better manage patients with advanced stage breast cancer, the discovery of novel biomarkers and therapeutic targets are both urgently required.

Extracellular vesicles or exosomes are secreted by different cell types and contain a wide range of cargoes, including nucleic acids (DNA, mRNA and non-coding RNA molecules), proteins (cytoskeletal proteins, cytokines and heat shock proteins), and enzymes such as GAPDH and ATPase [3]. Exosome has average diameter ranging from 30–150 nm [4]. Emerging evidence shows that protein such as growth factors and interleukin can be packaged into tumor-derived exosomes that regulates biological process in the recipient cells [5]. It has been shown that intracellular communications are accomplished through the exchange of exosomes in a variety of different cell types. Emerging evidence shows that exosomes are involved in many aspects of tumorigenesis including immune suppression, angiogenesis, cell migration and invasion [6–8]. Since exosomes can transfer signaling molecules to recipient cells (and vice versa) either within the tumor microenvironment or to distant sites [9], it is becoming clear that tumor-derived exosomes represent a powerful tool for cancer cells to promote malignant transformations in the normal cells. For instance, a recent study demonstrated that malignant breast cancer cells promoted metastasis through Annexin II enriched exosomes which promoted angiogenesis and created a more favorable tumor microenvironment [10]. More importantly, a newly discovered CBFB has been shown to be different roles in breast cancer.

CBFB (core binding factor subunit β) acts as transcription factor interact with RUNX protein activate the target genes that play important role normal development as well as human disease. The CBFB knocked down suppresses cell growth of the ovarian and prostate cancer. The CBFB inhibition in prostate xenograft mouse models decreased the tumor growth. Meanwhile suppressing the CBFB in ovarian xenograft mouse models improved the survival time [11]. In acute myeloid leukemia cells, upregulation of CBFB promote the resistant phenotype to a novel anti-leukemic therapy, RUNX1 inhibitor[12]. However, in breast cancer overexpression of CBFB suppress the Notch (oncogenic) signaling pathway and migration ability [13, 14]. In contrast, in other study revealed the CBFB upregulation is required for invasive ability of breast cancer cells[15]. In this discrepancy of CBFB role in breast cancer, our present study re-confirmed the role of CBFB in breast cancer. Further here we provided basis evidence of CBFB is enriched in breast cancer patient serum derived exosome.

The tumor microenvironment has long been considering facilitating the progression of breast cancer, especially in terms of distant metastasis. One of the most important players is cancer activated (or associated) fibroblasts (CAFs). Accumulating evidence indicates that CAFs secreted cytokines such as PDGF and TGF-β to recruit other fibroblasts and immune cells to promote angiogenesis and growth [16]. Similarly, exosomes are shown to be secreted within the tumor microenvironment for cellular communications between stromal cells and cancer cells to facilitate distant metastasis [1, 2]. Interestingly, co-cultured of exosome derived from breast cancer cells with normal fibroblast induced acquiring the cancer-associated fibroblast phenotypes [17]. However, the mechanism of exosome drives the cancer-associated fibroblast still poorly understood.
Base on the findings, tumor-derived exosomes may play essential roles in promoting breast cancer progression. In this study, we aimed to decipher the roles of exosomes in promoting bone metastasis and the contribution of CBFB that were found enriched in exosome in these processes. More importantly, we examined the effects of tumor derived CBFB-enriched exosomes on the generation of CAF-like phenotypes and their profound ability to promote metastatic potential. The findings may provide new insights for developing therapeutics against metastatic breast cancer patients.

Methods

Cell lines and cell culture

Clinical samples were obtained from patients with breast cancer. The procurement of the samples was strictly adherent to the approved IRB (No. N201603028) issued from Joint Institutional Review Board (JIRB) of MacKay Memorial Hospital. Non-tumor (NT) and tumor (T) tissues were collected from the MacKay Memorial Hospital breast cancer cohort. Human breast cancer cell lines MCF7 and T47D, both non-metastatic cell lines, and MDA-MB-157 and MDA-MB-436 representing metastatic triple-negative tumors, and human mammary fibroblast cell line, HMF3A, were all purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (DML10-1000ML, Caisson Labs, Smithfield, UT, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL Penicillin and 100 µg/mL Streptomycin, in 5% CO₂ humidified atmosphere at 37 °C. For maintenance, all cell lines were sub-cultured every 48–72 h.

CBFB-silencing and overexpression

Gene silencing experiments were performed using siRNA technique (Cat No. AM16708, CBFB siRNAs, ThermoFisher Scientific, USA). Lipofectamine 3000 (L3000008, Thermo Fisher Scientific, Waltham, MA, USA) was used for the transfection of the siRNAs following the manufacturer’s protocol. The overexpression experiments were carried out using Ultimate™ ORF Clones (Cat No. IOH46204, ThermoFisher Scientific, USA). The full-length CBFB fragment were subcloned to pcDNATM3.1 vector, using the Gateway® (Invitrogen) recombination cloning technology according to the protocls provided. CBFB silencing and overexpression were validated using western blots, anti-CBFB antibody (clone No. PA1-317, ThermoFisher Scientific, USA).

Exosome isolation

Sera from control health, primary breast cancer, and bone metastatic breast cancer patients were collected. Meanwhile breast cancer cell lines (negative control, siCBFB, and CBFB overexpressed cells) were cultured in serum-free medium for 48 h. Culture medium was collected. The Exosome were isolated from collected sera and culture medium from previously steps, and a standard procedure was performed [18]. Using repeated centrifugation and ultracentrifugation steps (500 × g for 10 min, 1200 × g for 20 min, and 10,000 × g for 30 min), followed by filtration with a 0.22 µm pore syringe and a spin at 100,000 × g for 60 min. The collected pellet was washed in PBS three times before another ultracentrifugation at 100,000
× g for 60 min. The exosomes were used for further analyses. Western blots with anti-CD63 (Cat No. H5C6, Novus Biologicals, Colorado, USA) and anti-CD9 (Cat No. 5G6, Novus Biologicals, Colorado, USA) antibodies were used to confirm the presence of exosomes.

**Cytokine estimation**

The concentration of IL-6 and OPN measured using sandwich enzyme immunoassay kits. (Cat. No. LS-F9982, LSBio, Seattle, USA and LS-F171, LSBio, Seattle, USA). The experiments were performed according to the user manuals provided. The concentration of IL-6 and OPN were determined by interpolation from a standard curve.

**Real-time PCR analyses**

To evaluate expression levels of genes of interest, real-time qPCR analyses were performed. Tumor cells or fibroblasts (2 × 10⁴) were co-incubated with exosomes (10 µg protein) for 48 h and cells were then harvested and lysed using RLT buffer (QIAGEN, Hilden, Germany). Total RNA was isolated by RNeasy Kit (QIAGEN, Hilden, Germany). cDNA was obtained using TaqMan in the StepOnePlus system (Applied BioSystems, Massachusetts, USA). All experimental Ct values were normalized against the Ct value of internal control, GAPDH. Relative abundance was determined by 2-ΔΔCt and expressed as fold changes. Primers used in this study are listed in Supplementary Table S1.

**SDS-PAGE and Western Blotting**

Total protein lysates were obtained and isolated from cancer cell lines using RIPA lysis buffer that supplemented with protease inhibitor (1x, Cat# 78430, Thermo Fisher Scientific, Waltham, MA, USA) and phosphatase inhibitor (0.5X, PierceTM Phosphatase Inhibitor Mini Tablets, Thermo Fisher Scientific, Waltham, MA, USA). BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine final protein concentration. The total protein sample were separated by standard SDS-PAGE using Protean III system (BioRad, California, USA) and transferred onto a PVDF membrane using Trans-Blot® Turbo™ Transfer System (BioRad, California, USA). The primary antibodies anti-Snail (Cat. No. 3895, 1:1000); anti-vimentin (Cat No. 5741, 1:1000); anti-CD44 (Cat No. 5640, 1:1000); anti-CXCR4 (Cat No. 97680, 1:1000) and anti-Runx2 (Cat. No. 12556, 1:500) were purchased from Cell Signaling Technology, MA, USA. Anti-OPN (Cat No. ab69498, 1:500) was purchased from abcam, Cambridge, UK. Primary antibodies used in this study are listed in Supplementary Table S2.

**Migration and invasion assays**

Invasion assays were performed in using the 24-well plate Transwell system (8-um pore-size) polycarbonate filter (#ECM508; Millipore, Massachusetts, USA). The lower chamber contained 400 µl of DMEM media supplemented with 10% FBS and 10 ng/ml rhEGF. The upper chamber was pre-coated with solubilized Matrigel (1 mg/ml, Corning, New York, USA) and seeded with 1 × 10⁴ breast cancer cells (0.1% FBS media) pre-incubated with or without Exo for 48 h. After 24 h cell incubation, media were discarded; cells on filter membrane were fixed with 4% formaldehyde for 1 h, and then stained with 0.1% (w/v) crystal violet solution, for 20 min, cells on the upper side of the inserts were removed. The cells were
evaluated as the total number on the lower surface of the membrane by microscopy. Migration assay performed following same steps, but without Matrigel on the upper chamber.

Flowcytometry

PE-VIM/α-SMA staining was used for detection of cell death using the BD FACSCanto™ II flow cytometry system (BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions. The cells were incubated with PE-labeled VIM and α-SMA at room temperature for 30 min and then analyzed using flow cytometry. The mitochondrial transmembrane potential (DΨm) was evaluated using the cationic dye JC-1 (Mitochondrial Membrane Potential Assay Kit, #ab113850, Abcam plc., Cambridge, UK) in accordance with the manufacturer's instructions (BD Pharmingen); 1 × 10^6 U87MG cells were incubated with 10 µg/mL JC-1 at 37 °C in the dark for 15 min, and then analyzed using flow cytometry. All samples were assayed three times in triplicate.

**In vivo analysis of CBFB-silenced cell lines-derived xenograft**

The animal experiments were performed strictly under the protocols approved by the MacKay Memorial Hospital (Affidavit of Approval of Animal Use Protocol #MMH-104-024). Tumor cells (control and CBFB-silenced, 2 × 10^6) were implanted subcutaneously in the right flank of NOD/SCID mice (6-week-old females, N = 5 per group) were purchased from the BioLASCO (BioLASCO Taiwan Co., Ltd. Taipei, Taiwan). Tumor growth was monitored on a weekly basis. The tumor volume was measured by a standard caliper and calculated using the following formula where tumor volume (V) = (W^2 × L)/2; W is the shortest diameter; L is the longest diameter of the tumor. The survival ratio of the two groups were tracked and represented analysis by Kaplan–Meier survival curve. After experiments, animals were humanely sacrificed by cervical dislocation and tumor samples were harvested for further analyses.

**Haematoxylin Eosin (H&E) staining**

Tissues were collected from the experimental animal's post sacrifice and fixed in 10% (vol/vol) formalin for 24 h and embedded in paraffin. Bones were decalcified prior to paraffin embedding. After de-waxing the paraffin-embedded 4 µm tissue sections using xylene for 2 min twice and re-hydrating with 100% ethanol twice for 2 min, 95% ethanol for 2 min, 75% ethanol for 2 min and ddH₂O for 2 min. The sections stained with hematoxylin for 2 min and wash in tap water for 10 min, counterstain in eosin for 30 s and Dehydrate with 75% ethanol for 30 s twice, 95% ethanol for 30 s, 100% ethanol and xylene for 30 s. The sections were mounted with mounting medium and examined under a light microscope and the tumor areas were digitally photographed. The tumor areas were calculated using Image J software.

**Statistical analysis**

Kaplan Meier overall survival analysis were performed using GraphPad Prism for Windows version 7.00 software (GraphPad Software Inc., San Diego, CA, USA). All the experiments in this study were performed at least three times in triplicates and results expressed as Mean ± SD. Quantitative analyses of migration
and invasion assays data were performed using ImageJ version 1.5 (Wayne Rasband National Institutes of Health, Bethesda, MD, USA) The Student-T test was used to determine the statistical difference between control and experimental group. The One-way analysis of variance (ANOVA) was used to compare ≥ 3 groups. \( p \)-values (represented by asterisks), where *\( p < 0.05 \); **\( p < 0.01 \) and ***\( p < 0.001 \).

## Results

**CBFB is up regulated in TNBC cells and correlated with a poor prognosis**

Using TCGA databases [19, 20], we first identified that a higher CBFB expression was significantly correlated with a shorter metastasis-free survival time in patients with breast cancer (Fig. 1a). Similarly, CBFB mRNA level was profoundly correlated to a higher risk for breast cancer (Fig. 1b). We found the human mammary fibroblast cell line (HMF3A) and normal like breast cell lines (MCF10A) have no difference in CBFB expression level. Markedly, the low-metastatic potential breast cancer cell lines (MCF7 and T74D) expressed the same level of CBFB relative to MCF10A. Interestingly, we showed that metastatic breast cancer cell lines, MDA-MB-436 and MDA-MB-157 contained a significantly higher level of CBFB as compared to low-metastatic potential counterparts, MCF7 and T47D (Fig. 1c). In consistency, CBFB expression data from the CCLE showed the primary breast cancer cell lines have significant difference to metastatic breast cancer cell lines (Supplementary Fig. S1). However, markedly we found two cell lines derived from brain and skin metastatic breast cancer were the most CBFB over expressed relative all breast cancer cell line (primary and metastatic derived breast cancer cell lines). To explore the importance of CBFB in metastatic breast cancer, we assessed the CBFB expression from primary, lung, brain, and bone metastatic breast cancer tissues. We observed that the lung metastatic and primary breast tumor tissues have no significant different in CBFB level expression. Meanwhile the bone and brain metastatic breast cancer tissues obviously expressed CBFB higher than primary and lung metastatic breast cancer tissues. Consistent to the cancer cell line data that the CBFB expression levels depend on the tissue dependent manner (Fig. 1d).

Serum exosomes enriched with CBFB promotes metastatic potential of breast cancer cells.

Serum exosomes (Exos) have been extensively explored for their roles in prognosis in different diseases including breast cancer [9, 21]. We obtained serum exosomes from control health, primary breast cancer patients, and bone-metastasis breast cancer for analyses. Markedly, the isolated exosome from bone metastasis breast cancer patients expressed higher CBFB than the exosome isolated from control health and primary breast cancer patients (Fig. 2a). Low metastatic potential breast cancer cell lines, T47D and MCF7 were incubated with exosome derived from bone metastatic breast cancer patients and showed a significantly increased migration (Fig. 2b) and invasion (Fig. 2c). This exosome-mediated increased metastatic potential in low metastatic potential breast cancer cells was reflected by the upregulation in CBFB and Runx2, EMT markers including Snail, CD44, and bone-metastasis markers such as OPN, at both mRNA and protein levels (Fig. 2d).
CBFB enriched Exos promotes the generation of cancer-associated fibroblast-like phenotype (CAF-like phenotype).

The tumor microenvironment plays a central role for disease progression. In particular, cancer-associated fibroblasts (CAFs) have been shown to be a key player for promoting distant metastasis and drug resistance of breast cancer cells [16]. Here, we showed that a significantly increased level of α-SAM, FAP and vimentin (all markers of CAFs) while decreased level of cytokeratin 1 (KRT1) was observed in the mammary fibroblast cell line, HMF3A cells, after treated with Exos (Fig. 3a). To assess the proportion of CAF-like cells we performed flowcytometry targeting Vimentin and α-SAM. Markedly, the Exo treatment increased the CAF-like (VIM+/α-SAM+) cell proportions (Fig. 3b). The western results revealed that HMF3A-Exo treated expressed higher CBFB than the untreated HMF3A cells (Fig. 3c). These findings suggest the CBFB enriched exosome were up taken by HMF3A as source of CBFB drive the CAF-like phenotype acquiring. Next, we co-cultured CAF-like cells (generated from HMF3A cells by incubation with Exos), with T47D and MCF7 cells. We observed significantly enhanced metastatic potential in both cell lines as reflected by their increased migratory (Fig. 3d) and invasive (Fig. 3e) abilities. In support, an increased secretion of IL-6, OPN by the CAFs into the culture medium was observed (Fig. 3f).

CBFB level was associated with the metastatic potential in breast cancer cells.

Subsequently, we investigated the metastasis-regulating function of CBFB using gain-of-function and loss-of-function methodology. First, we showed that CBFB-silenced MDA-MB-436 and MDA-MB-157 cells showed a significantly reduced metastatic ability, as reflected by the prominently reduced migratory and invasive ability (Fig. 4a); CBFB-silenced MDA-MB-436 and MDA-MB-157 cells showed a markedly down-regulated EMT markers, Snail, vimentin, CD44, and bone-metastasis associated markers, OPN and Runx2 (Fig. 4b). In parallel, we overexpressed CBFB in low metastatic potential T47D and MCF7 cells. CBFB-overexpressing T47D and MCF7 showed significantly increased migratory and invasive abilities as compared to their parental counterparts (Fig. 4c). In support, CBFB-overexpressing T47D and MCF7 cells also exhibited a substantially increased EMT markers such as Snail, vimentin, CD44, OPN and Runx2 (Fig. 4d). Interestingly, we also compared the Exos released into the culture media of CBFB-silenced MDA-MB-436 and their parental cells.

CBFB-silencing significantly reduced metastasis in MDA-MB-436-derived xenograft model.

To validate our data obtained from in vitro experiments, we used MDA-MB-436-derived xenograft mouse model were orthotopically injected into the mammary fat pad of NOD-SCIC female mice. MDA-MB-436-derived xenograft mice injected with CBFB-silenced cells clearly showed a lower tumor burden over time as compared to control group (Fig. 5a). For instance, at week 4, the tumor size of CBFB-silenced mice was significantly smaller as compared to the control group (Fig. 5a). Mice bearing CBFB-silenced tumor cells showed a significantly longer survival rate (Fig. 5b). In subsequent experiments using tumor sample derived from the CBFB-silenced mice, we demonstrated that compared to the control group, CBFB, OPN Runx2 and CXCR4 protein expression levels were significantly suppressed (Fig. 5c). More importantly, we compared the pooled sera of the two groups and found that serum exosomes from CBFB-silenced mice
contained a significantly lower amount of CBFB (Fig. 5d); in addition, the circulating serum level of Runx2, IL-6, CXCR4, and OPN were also significantly lower in the CBFB-silenced group (Fig. 5e) and Summarized schema in this study of Fig. 5f.

**Discussion**

Bone is one of the most sites for breast cancer cells to metastasize. Although a significant advance in therapeutic development in the past decade, there is limited option for treating patients with breast to bone metastasis and the prognosis remains poor for these patients. Therefore, new and/or alternative treatment options are urgently required. In order to develop more effective treatment strategies against breast to bone metastasis, a better understanding of the underlying mechanisms is essential. In this study, we first recognized an elevated oncogene CBFB was associated with poor survival ratio in TNBC patients. Previous studies have demonstrated that increased CBFB activity was associated with the increased invasiveness in breast cancer cells [15, 22]. This is in line with our observation where an increased CBFB was positively correlated with poor prognosis in the patients with triple-negative phenotype. In addition, CBFB expression was found to be significantly higher in the metastatic breast cancer cell lines such as MDA-MB-157 and MDA-MB-436 as compared to their low metastatic potential counterparts, MCF7 and T47D cells (Fig. 1). In terms of clinical relevancy, we found that CBFB mRNA level was significantly higher in the patient samples of bone-metastasis as compared to their non-metastatic counterparts as well as the lung metastatic and brain metastatic.

To explore further, we demonstrated that the serum exosomes (Exo) from patients with bone-metastasis could significantly promote the metastatic potential in non-metastatic breast cancer cell lines. We found that after cultured with exosomes, both MCF7 and T47D cells showed significantly increased mobility in concert with increased expression of EMT markers such as Snail and CD44; an increased expression of Snail has been shown to be essential for initiating EMT process in many different cancer types including breast [3, 8]; an increased CD44 expression has been used as a marker as metastasis in breast cancer cells as well as a marker for cancer stem cells [6, 7]. In addition, OPN and Runx2 both involved in the shaping of tumor microenvironment for bone metastasis [23], were also elevated in exo-treated MCF7 and T47D cells. Notably, we detected an elevated level of CBFB in the serum exosomes isolated from patients with bone metastasis versus their non-metastatic, lung metastatic, and brain metastatic counterparts (Fig. 2). These findings strongly suggested that serum exosomes from patients with metastatic phenotype contained metastasis and oncogenic signaling molecules and play an essential role in transforming non-metastatic breast cancer cells into metastatic lineage.

In parallel, we also examined the transforming effects of exosomes on normal fibroblasts. We found that Exo-treated fibroblasts showed a significantly increased makers of CAFs such as vimentin and α-SAM. Functionally, these exo-treated fibroblasts secreted an increased amount of OPN and IL-6 into the culture medium as compared to the normal fibroblasts. Both OPN and IL-6 have been supported in literature where they promoted oncogenic and metastatic signaling pathways in different cancer types [24, 25]. An increased OPN in serum has been found in patients with metastatic cancer [26] and associated with
higher bone metastatic frequency [27, 28]. Therefore, our findings may add another level of understanding into as how bone metastasis can be promoted by way of tumor derived exosomes. In addition, tumor-derived exosomes also contribute to transform the stromal fibroblasts to CAF-like phenotype cells (Fig. 3).

We further demonstrate the function of CBFB by silencing its expression in metastatic MDA-MB-436 cells and overexpressing CBFB in non-metastatic T47D cells. Indeed, we observed the silencing of CBFB significantly reduced both the migratory and invasive abilities in MDA-MB-436 cells while prominently increased these abilities in the T47D cells (Fig. 4). In support, a previous study showed that Runx2 and CBFβ are essential to regulate genes which enable metastatic breast cancer cells to directly modulate the functions of osteoblasts and osteoclasts [22]. More importantly, we found that silencing CBFB in MDA-MB-436 cells resulted in the reduced expression of EMT regulator Snail, metastasis/cancer stem cell marker, CD44, and bone metastasis associated markers OPN and Runx2. Furthermore, the exosomes released from CBFB-silenced MDA-MB-436 and MDA-MB-157 cells contained a lower level of CBFB whereas the opposite was true in the CBFB-overexpressing T47D and MCF7 cells. This finding indicated the total cellular CBFB mRNA level might dictate the amount of CBFB packaged into exosomes. Further investigation is currently under investigation in our laboratory.

Finally, we showed that CBFB-silencing resulted in the significantly reduced tumor burden as well as the incidence of bone-metastasis and increased survival rate in MDA-MB-436-derived xenograft mouse model. In addition, the reduction in CBFB in the serum exosomes was reflective of our cellular study. More importantly, a significantly reduced level of CXCR4, OPN, Runx2 and IL-6 was detected in the CBFB-silenced tumor samples as compared to their control counterparts (Fig. 5). A wealth of evidence supports the metastasis-promoting function of CXCR4 signaling [29, 30]. An increased level of OPN in tumor cells and tissues has been shown to predict a higher incidence of bone metastasis [31]. Similarly, Runx2 represents another key bone-metastasis marker in breast cancer. An increased Runx2 expression or activity was linked to the increased TGF-β signaling and enhanced bone colonization of breast cancer cells [32–34]. Interestingly, the observation where CBFB-silencing led to the decreased expression of all these bone-metastasis associated factors might implicate that CBFB as a novel drug target for bone metastasis in breast cancer patients. Also, our findings suggested that these molecules might be used collectively as a prognostic signature for bone metastasis and survival in the clinical settings in the future. However, a much larger samples will be required to validate our hypothesis.

**Conclusion**

In conclusion, as shown schema abstract in Fig. 6 we demonstrated the essential roles of CBFB in the promotion of bone metastasis in breast cancer cells. In addition, CBFB appeared to be enriched in the tumor derived exosomes or serum exosomes of patients with bone metastasis. The suppression of CBFB led to the decreased tumor burden and bone metastasis in association with decreased markers of bone metastasis including CXCR4, Snail, CD44, OPN, Runx2 and IL-6.
Declarations

Ethics approval and consent to participate

Clinical samples were obtained from patients with breast cancer. The procurement of the samples was strictly adherent to the approved IRB (No. N201603028) issued from Joint Institutional Review Board (JIRB) of MacKay Memorial Hospital. Non-tumor (NT) and tumor (T) tissues were collected from the MacKay Memorial Hospital breast cancer cohort.

Consent for publication

The authors declare that they have no potential financial competing interests that may in any way, gain or lose financially from the publication of this manuscript at present or in the future. Additionally, no non-financial competing interests are involved in the manuscript.

Availability of data and materials

The datasets used and analyzed in the current study are publicly accessible as indicated in the manuscript.

Conflict of Interests: The authors declare that they have no potential financial competing interests that may in any way, gain or lose financially from the publication of this manuscript at present or in the future. Additionally, no non-financial competing interests are involved in the manuscript.

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Authors’ contributions: Chih-Ming Su\textsuperscript{1,2#}, Yu-Hsin Lai\textsuperscript{3,4#}. Study conception and design, collection and assembly of data, data analysis and interpretation, and manuscript writing. Oluwaseun Adebayo Bamodu\textsuperscript{5}, Kuang-Tai Kuo\textsuperscript{6,7}, Iat-Hang Fong\textsuperscript{5}: Data analysis and interpretation. Chi-Tai Yeh\textsuperscript{5,8}, Wei-Hwa Lee\textsuperscript{9*} and Wen-Chien Huang\textsuperscript{10,11*}: Study conception and design, data analysis and interpretation, final manuscript approval. All authors read and approved the final manuscript.

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**Figures**
Increased CBFB expression is associated with metastasis and poor prognosis in breast cancer patients. 

a A Kaplan-Meier metastasis-free survival curve derived from TCGA breast cancer cohort (N=776) implicates a shorter metastasis-free survival time for the patients with a higher level of CBFB.  
b Oncomine database analysis depicts CBFB mRNA level was significantly higher in the patients with high risk for breast cancer.  
c Comparative qPCR analysis of CBFB expression between immortalized human mammary fibroblast (HMF3A), normal-like breast (MCF10A), low metastatic potential (MCF7, T74D), and metastatic breast cancer (MDA-MB-436, MDA-MB-157) cell lines. We found the CBFB mRNA expression level in HMF3A, MCF10A, MCF7, and T74D were not significant different. CBFB mRNA level was found
significantly higher in both metastatic cell lines, MDA-MB-436, MDA-MB-157 as compared to normal-like breast cell line (MCF10A) and low metastatic potential MCF7 and T47D counterparts. d Differential CBFB mRNA expression between non-metastasis versus bone-metastasis samples. A significantly highest level of CBFB was observed in the clinical samples from patients with bone-metastasis among all samples. Each group consists of 10 samples. NS (not significant); **p<0.01; ***p<0.001.

Figure 3
Exosomes enriched with CBFB promoted the generation of CAF-like phenotype. a HMF3A cells treated with CBFB enriched exosome derived from bone metastatic breast cancer patients (Exo) showed a significantly increased CAF markers including vimentin (Vim), FAP, α-SAM, and decreased cytokeratin (KRT1) of immortalized human mammary fibroblast cell line (HMF3A). b The CAF-like (VIM+/αSMA+) cell percentages was increased following the Exo treatment. c The western results demonstrated the upregulation of CBFB in the HMF3A cells treated with Exo. HMF3A-Exo co-culture with low metastatic potential breast cancer cell lines enhanced metastatic potential. Both low metastatic potential breast cancer cell lines, MCF7 and T47D cells showed enhanced migratory d and invasive e abilities as compared to their control counterparts. Representative images of invasion are shown. Scale bars: 200 μm. f Elisa analyses of Exos-induced CAFs. CAFs generated from Exos co-culture demonstrated an increased secretion of IL-6 and OPN into the culture medium. **p<0.01; ***p<0.001.

Figure 4

CBFB expression was associated with metastatic potential of breast cancer cells. a Highly metastatic MDA-MB-436 cells were subjected to CBFB-silencing. CBFB-silenced MDA-MB-436 cells (Si) showed a significantly reduced migratory (left panels) and invasive (right panels) abilities. The insert represents the western blots indicating CBFB was silenced as compared with negative control (NC). b Western blots demonstrate the significantly reduced expression of Snail, vimentin (Vim), OPN, CD44 and Runx2 in...
CBFB-silenced MDA-MB-436 cells as compared to their parental counterparts (NC). c Non-metastatic T47D cells were overexpressed with CBFB. CBFG-overexpressing (OE) T47D cells showed an enhanced migratory (left panels) and invasive (right panels) abilities as compared to their parental counterparts (NC). Representative images of invasion are shown. Scale bars: 200 μm. d Western blots comparing parental and CBFB-overexpressing T47D cells. Metastasis associated markers Snail, CD44, vim, OPN and Runx2 were all elevated in the OE cells. ***p<0.001.

Figure 6

Metastatic breast cancer cells secrete exosomes enriched with CBFB and facilitate the transformation of cancer associated fibroblasts-like phenotype cells (CAF-like phenotype cells). CAF-like cells and exosomal-CBFB in turn promote epithelial-to-mesenchymal (EMT) transition of breast cancer cells, resulting increased bone-metastasis incidence.