

# Dormancy and growth of metastatic breast cancer cells in a bone-like microenvironment

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Received: 23 December 2014 / Accepted: 28 February 2015  
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**Abstract** Breast cancer can reoccur, often as bone metastasis, many years if not decades after the primary tumor has been treated. The factors that stimulate dormant metastases to grow are not known, but bone metastases are often associated with skeletal trauma. We used a dormancy model of MDA-MB-231BRMS1, a metastasis-suppressed human breast cancer cell line, co-cultured with MC3T3-E1 osteoblasts in a long term, three dimensional culture system to test the hypothesis that bone remodeling cytokines could stimulate dormant cells to grow. The cancer cells attached to the matrix produced by MC3T3-E1 osteoblasts but grew slowly or not at all until the addition of bone remodeling cytokines, TNF $\alpha$  and IL- $\beta$ . Stimulation of cell proliferation by these cytokines was suppressed with indomethacin, an inhibitor of cyclooxygenase and of prostaglandin production, or a prostaglandin E2 (PGE2) receptor antagonist. Addition of PGE2 directly to the cultures also stimulated cell proliferation. MCF-7, non-metastatic breast cancer cells, remained dormant when co-cultured with normal human osteoblast and fibroblast growth factor. Similar to the MDA-MB-231BRMS1 cells, MCF-7 proliferation increased in response to TNF $\alpha$  and IL- $\beta$ . These findings suggest that changes in the bone microenvironment due to inflammatory cytokines associated with bone repair or excess turnover may trigger the occurrence of latent bone metastasis.

**Keywords** Breast cancer · Dormancy · Three-dimensional bioreactor · Bone metastases · Prostaglandins

## Introduction

The 5 year cure rate for localized breast cancer is high, e.g. 99 % [1]. However, this figure belies the fact that breast cancer can reoccur as metastatic disease many years and even decades after the original treatment [2, 3]. Once relapse occurs, and the cancer colonizes in distant organs, the relative survival rate drops to 24 % [1]. One of the preferred metastatic sites for breast cancer is the skeleton. It is estimated that 65–75 % of individuals with advanced disease harbor bone metastases [4], and that over 70 % of patients dying from breast cancer have evidence of bone metastases at post-mortem examination [5]. In fact, it has been suggested that many patients have undetected disseminated tumor cells (DTC) or micro-metastases at the time of diagnosis of the primary tumor [6]. There is evidence that the process of primary tumor resection may trigger metastasis [7]. Indeed, the bone may provide a transient niche from which metastatic cells may later seed other secondary organs [8].

Not all DTC that lodge in secondary organs will grow. The efficiency of metastasis is estimated to be low [9]. Dissemination alone is not sufficient to cause formation of "...overt, vascularized, clinically detectable metastases" [10, 11]. Cancer cells can remain dormant in secondary organs for long periods, often years or even decades depending on the tumor [2, 12]. There are reports of the transfer of DTC to patients through organs transplanted from individuals either not known to have cancer or thought to be cured for many years. These occult DTC then grew in the immunosuppressed recipient (see [2]). It is

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estimated that 30 % of breast cancer patients diagnosed at the MO tumor-node-metastasis tumor stage already contain DTC in their bone marrow [10]. Dormant cells apparently survive chemotherapy, radiation and adjuvant therapy, and may reawaken at a later time and proliferate as bone metastases. The prediction of metastatic recurrence is poor at best. Current estimates are based on the phenotype of the tumor and use of circulating tumor cells (CTC) as prognostic indicators [13, 14]. The numbers of these CTC as well as their gene signatures are being used to develop predictive algorithms. However, the results are far from definitive [15]. Identification of the factors that either maintain the dormant state or cause dormant cells to proliferate is crucial to the development of clinical strategies to prevent recurrence of malignancy.

There is evidence that disruption of the dormant tumor cell niche may trigger recurrence of dormant cells many years after primary treatment. Local trauma, wounding, or injury may spur tumor cells to grow. Chronic inflammation and/or immunosuppression also are important factors to be considered in cancer recurrence (reviewed by [6]). Das Roy et al. found an increase in lung and bone marrow metastasis using an arthritic mouse model [16]. Recently, Yano [17] reported the case of a woman who experienced breast cancer relapse 24 years after mastectomy and radiation treatment, when administered drugs for rheumatoid arthritis. In another case, [18] a tracheostomy wound was the site of breast cancer outgrowth for a woman 10 years after mastectomy. In fact, it was recognized over a century ago that the surgical process employed to remove the primary tumor might itself promote metastasis (reviewed by [19]). There is also evidence that recombinant PTH (aa 1-34) which enhances bone turnover, also causes increased bone metastasis in rodents and possibly in humans [20, 21].

Given this anecdotal evidence, we speculated that cytokines involved in bone remodeling and repair post trauma [22] play a role in the growth of dormant breast cancer cells in the bone. For this study, we used a specialized three-dimensional (3D) model of an *in vitro* bone mimic that permits the growth of a multiple layer of mineralized osteoblast tissue from pre-osteoblasts [23]. We had observed that a human metastatic breast cancer cell line, MDA-MB-231 [24], grows in this chamber in a manner that mimics metastatic breast cancer growth in bone [25]. However, a metastasis suppressed variant, MDA-MB-231BRMS1 [26], does not readily grow in this same bone-like environment [27]. The BRMS1 variant shows this same property in mice; i.e. in an experimental model of metastasis, the BRMS1 cells are detected in the bone marrow but seldom grow there [28, 29]. The weakly metastatic human breast cancer cell line, MCF-7, has also been used as a model for breast cancer cell dormancy [30]. In this model, the addition of fibroblast growth factor (FGF) to MCF-7 cells

grown on matrigel causes the cells to enter a state of dormancy.

In the bone microenvironment, cytokines play a vital role in bone turnover, remodeling, and repair. Transforming growth factor  $\alpha$  (TNF $\alpha$ ), interleukin 1 $\beta$  (IL-1 $\beta$ ) and interleukin 6 (IL-6) are reported to be key signaling molecules in the multistep process of bone remodeling [20]. Furthermore, TNF $\alpha$  and IL-1 $\beta$  are known to stimulate production of prostaglandin E2 (PGE2), an important inflammatory molecule, in numerous cell types including osteoblasts [31, 32]. PGE2, in turn, is known to upregulate the production and phosphorylation of focal adhesion kinase (FAK) [33] which plays a key role in cell adhesion, motility and survival.

In our current study, addition of a cocktail of bone remodeling cytokines to the 3D dormancy model cultures resulted in a marked increase in proliferation of the breast cancer cells in both culture systems. The proliferative effect was also seen with the addition of exogenous PGE2. However, a dormant state was maintained in the presence of the cytokines with the addition of indomethacin, a COX inhibitor or AH6809, a PGE2 receptor antagonist. Increased formation of focal adhesion kinase plaques by the cancer cells treated with bone remodeling cytokines was also observed.

## Materials and methods

### Cell culture

The human metastatic breast cancer cell line, MDA-MB-231 [24] and its metastasis-suppressed variant, MDA-MB-231BRMS1 [26] were gifts of Dr. Danny Welch, University of Kansas Cancer Center. MDA-MB-231 cells were cultured in DMEM (Corning Cellgro, Manassas, VA), 5 % fetal bovine serum (PAA Laboratories, Etobicoke, Ontario), 1 % non-essential amino acids (Corning Cellgro) and penicillin/streptomycin (Corning Cellgro) at 100 IU/mL and 100 mg/mL concentration, respectively. MDA-MB-231BRMS1 cells were grown in DMEM/F12, 5 % FBS, 1 % NEAA and penicillin/streptomycin. The cell line, MCF-7 [30], was a gift from Dr. Robert Wieder, Rutgers University, and was propagated in DMEM, 10 % FBS, penicillin/streptomycin. All cancer cell lines were engineered to express green fluorescent protein (GFP). The murine osteoblast precursor cell line, MC3T3-E1 [34], was provided by Dr. Norman Karin, University of Texas, and was propagated in  $\alpha$  MEM (Corning Cellgro), 10 % FBS and penicillin/streptomycin. In order to differentiate the osteoblasts, 10 mM  $\beta$ -glycerophosphate and 50  $\mu$ g/mL ascorbic acid were added to the medium. Normal human osteoblasts, NH Ost, and the proprietary growth and differentiation media were purchased

from Lonza (Walkersville, MD) and grown according to their protocol.

Osteoblast bioreactor cultures were established as previously described [23]. In brief, 10,000 cells/cm<sup>2</sup> of either MC3T3-E1 or NHOst were seeded in the growth chamber of the bioreactor in the appropriate differentiation medium containing either 10 % (MC3T3-E1) or 15 % (NHOst) fetal bovine serum. The upper medium reservoir was filled with differentiation medium without serum and was replaced every 2–3 weeks. MC3T3-E1 cultures were maintained for 2 months; NHOst cultures for 1 month.

### Cytokine and cancer cell addition

All cytokines and neutralizing antibodies were purchased from R&D Systems (Minneapolis, MN). PGE2 was obtained from Cayman Chemical (Ann Arbor, MI). The bone remodeling cytokine cocktail for the MC3T3-E1/BRMS1 dormancy model initially consisted of TNF $\alpha$  (5 ng/mL), IL-1 $\beta$  (10 ng/mL), IL-6 (10 ng/mL) and PGE2 (100 nM), but was later reduced to TNF $\alpha$  and IL-1 $\beta$ . The remodeling cytokines for the NHOst/MCF-7 model was composed of TNF $\alpha$  (5 ng/mL), IL-1 $\beta$  (10 ng/mL), IL-6 (10 ng/mL), IL-8 (0.5 ng/mL) and MCP-1(2 ng/mL). In addition, 10 ng/mL basic fibroblast growth factor (bFGF) was added to the NHOst/MCF7 cultures to establish dormancy. For neutralizing antibody experiments, anti-human TNF $\alpha$ , IL-1 $\beta$ , and IL-6 were added at concentrations of 5, 20, and 0.6  $\mu$ g/mL, respectively. Cytokines and neutralizing antibodies were added to the growth chamber only. The cyclooxygenase inhibitor, indomethacin, purchased from Sigma-Aldrich (St. Louis, MO), was added to both chambers of the bioreactors at a concentration of 50  $\mu$ M. The PGE2 receptor antagonist, AH6809, was obtained from Cayman Chemical and used at a concentration of 50  $\mu$ M in both reactor chambers.

Cytokines and inhibitors were added to the bioreactor osteoblast cultures. Approximately 15 min after their addition, the MDA-MB-231 or MDA-MB-231BRMS1 cells at a concentration of 4000 cells/cm<sup>2</sup> were added to the mature murine osteoblast cultures; MCF-7 cells were added to the NHOst cultures at a concentration of 2000 cells/cm<sup>2</sup>. The medium in the upper reservoir was replaced with fresh differentiation medium at the same time.

### Live cell imaging

Bioreactor cultures were imaged daily for 3–4 days of the co-culture period using the Olympus FV300 confocal microscope at a 200 $\times$  magnification. Three to six representative images were captured for each bioreactor culture at each time point. Images were analyzed by ImageJ [35] using area fraction quantitation methodology. Statistical

analyses were performed with GraphPad Prism 4.0 using two-way ANOVA with Bonferroni correction.

### Prostaglandin E2 assay

The level of PGE2 in the bioreactor culture supernatants was measured by a competitive enzyme immunoassay method (GE Healthcare, Piscataway, NJ).

### Immunocytochemistry

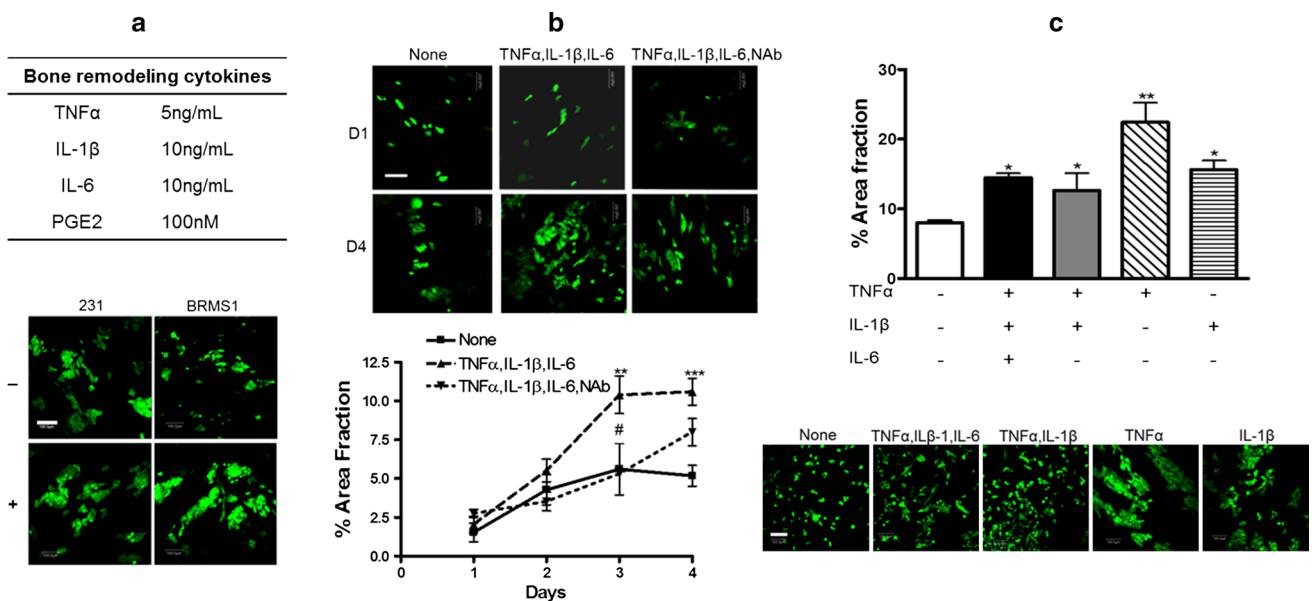
After 3–4 days of co-culture, the bioreactors were disassembled. The growth chamber membrane with attached cells and matrix was carefully excised from the device and rinsed once with PBS. The membrane was then fixed in 4 % paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and stored at 4 °C. Culture membranes were divided into small portions for immunostaining.

The primary rabbit antibodies to Ki67 (ab927442) and focal adhesion kinase (phospho Y397; ab4803) were purchased from Abcam (Cambridge, MA). A goat anti-rabbit IgG antibody conjugated to Alexa 568 (Life Technologies, Grand Island, NY) was used for detection. Briefly, membrane fragments were rinsed in PBS. Cells were permeabilized in 0.05 % triton X-100 in PBS for 15 min then washed in PBS and blocked in PBS containing 10 % normal goat serum (NGS) for 1 h. Antibodies for Ki67 and FAK were diluted in PBS 1 % NGS at 1:300 and 1:100, respectively, and applied to the membranes for 2 h. After washing the membranes three times with PBS, the secondary goat anti-rabbit IgG Alexa 568 was diluted 1:200 in PBS 1 % NGS and applied for 1 h. Membranes were washed 3 times in PBS and mounted on glass slides with Fluoromount G (Southern Biotech, Birmingham, AL). Slides were imaged using a Keyence BZ-X700 fluorescence microscope with 60 $\times$  and 100 $\times$  lenses.

## Results

### Bone remodeling cytokines stimulated the proliferation of MDA-MB-231BRMS1 cells

A cocktail of cytokines reported to be present during bone remodeling [22, 36, 37], TNF $\alpha$  (5 ng/mL), IL-1 $\beta$  (10 ng/mL), IL-6 (10 ng/mL) and PGE2 (100nM), was added to 2 month old bioreactor cultures of MC3T3-E1 (Fig. 1a). Approximately 15 min later MDA-MB-231<sup>-GFP</sup> or MDA-MB231BRMS1<sup>-GFP</sup> cells were added to the cell growth chambers. The co-cultures were examined daily by confocal microscopy for 4 days. As seen previously, the cells attached to the matrix. The cytokine treatment had no obvious effect on the growth or appearance of the 231 cells. However, the



**Fig. 1** Bone remodeling cytokines increased the growth and affected the morphology of MDA-MB-231BRMS1 breast cancer cells grown in a 3D osteoblast culture. The bone remodeling cytokine cocktail, consisting of TNF $\alpha$ , IL-1 $\beta$ , IL-6, and PGE2, was added to a 2 month old, 3D culture of MC3T3-E1 murine osteoblasts 15 min prior to the addition of  $10^5$  MDA-MB231 $^{GFP}$  or MDA-MB-231BRMS1 $^{GFP}$  human breast cancer cells. Live images of the co-cultures were collected daily using confocal microscopy. Image quantitation was performed using ImageJ; statistical analysis was performed using GraphPad Prism. **a** Representative images of day 4 co-cultures of 231 or BRMS1 cells with and without the bone remodeling cytokine

cocktail ( $n = 3$ ). **b** Images and area fraction graph of BRMS1 osteoblast co-cultures incubated with TNF $\alpha$ , IL-1 $\beta$ , and IL-6 with and without addition of neutralizing antibodies (NAb) to the three cytokines. Shown are representative images from days 2 and 4 of co-culture ( $n = 3$ ). **c** Representative day 3 images and area fraction analysis of BRMS1 co-cultures with TNF $\alpha$  and/or IL-1 $\beta$  additions ( $n = 3$ ). Scale bar 100 microns. \*\*\* $p = 0.001$ ; \*\* $p = 0.01$ ; \* $p = 0.05$  when comparing cultures with and without cytokines. # $p = 0.01$  when comparing cultures with cytokines and NAb.  $n = 3$

BRMS1 cells grew into large multicellular colonies in comparison to little or no growth in the absence of added cytokines (Fig. 1). This growth pattern was similar to that observed for untreated metastatic 231 cells.

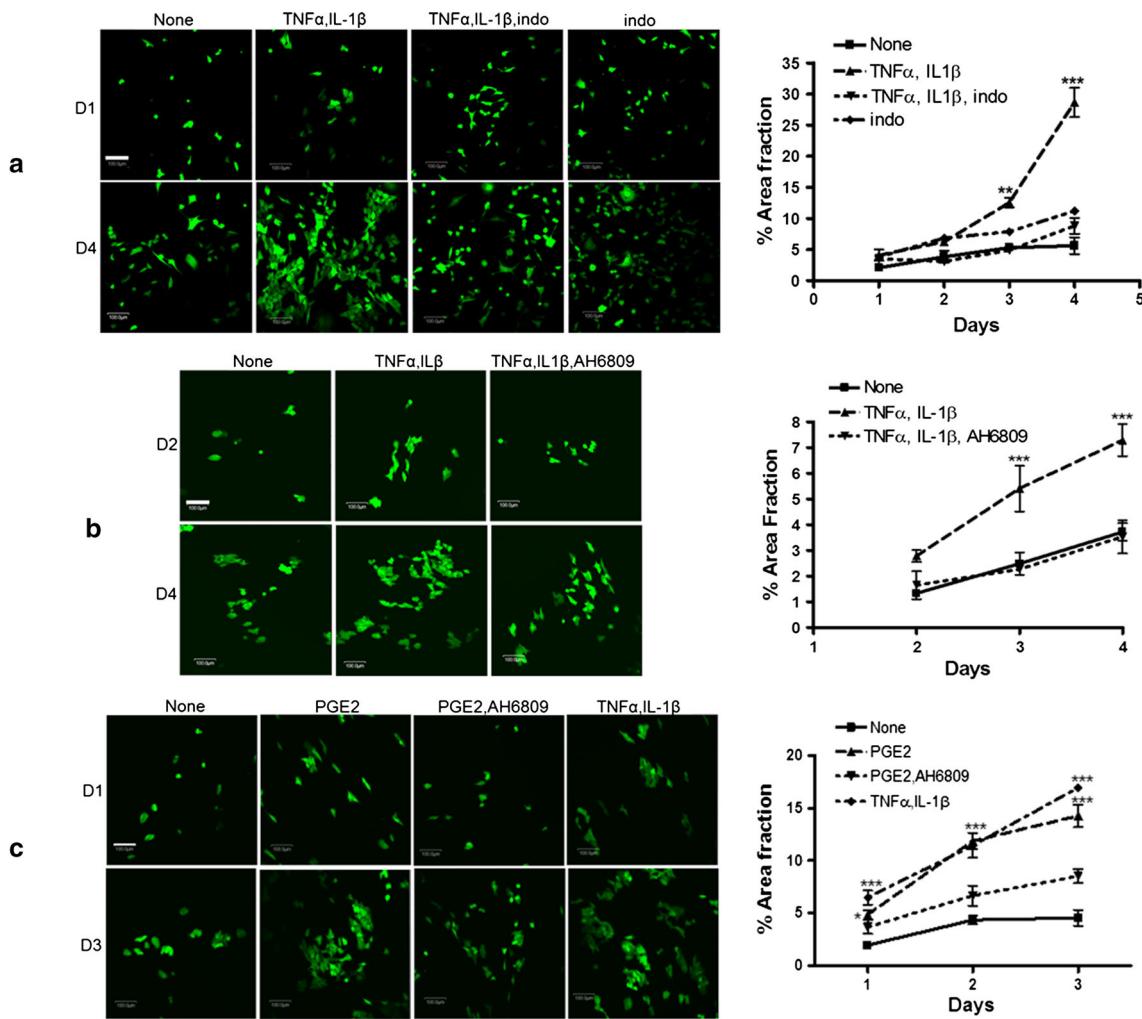
Because TNF $\alpha$ , IL-1 $\beta$ , and IL-6 can activate the arachidonic acid pathway leading to production of prostaglandins [38–40], we omitted PGE2 from the cocktail and repeated the experiment (Fig. 1b). Over four days of culture without added cytokines, there was a small increase in the area fraction occupied by the BRMS1 cells indicative of slow growth over time. Addition of TNF $\alpha$ , IL-1 $\beta$ , and IL-6 was sufficient to enhance the growth of the BRMS1 cells without the inclusion of PGE2. By day four of culture, the cells had increased more than twofold over those with no cytokines added. The addition of neutralizing antibodies (Nab) to TNF $\alpha$ , IL-1 $\beta$  and IL-6 at the beginning of the co-culture period prevented the increase in proliferation elicited by the cytokines, at least for the first 3 days of culture (Fig. 1b).

In order to further narrow down the list of effector cytokines, we tested TNF $\alpha$  and IL-1 $\beta$  in tandem and individually. We had previously observed that IL-6 had no effect on BRMS1 growth (data not shown). TNF $\alpha$  and IL-1 $\beta$  alone or together caused an increase in BRMS1 colony formation

(Fig. 1c). The increase in growth compared to cultures without cytokines ranged from two to four fold.

### Prostaglandin E2 was the effector molecule

Because both TNF $\alpha$  and IL-1 $\beta$  can initiate the arachidonic acid pathway, we asked if indomethacin, an inhibitor of COX1 and COX2, could block the growth response of BRMS1 cells to these cytokines. In this set of experiments, TNF $\alpha$  and IL-1 $\beta$  increased growth of BRMS1 cells in the cultures by over sevenfold at day four when compared to cells grown without added cytokines (Fig. 2a). The addition of 50  $\mu$ M indomethacin prevented the cytokine-induced increase. Indomethacin alone did not affect cell growth (Fig. 2a). Interestingly, indomethacin also appeared to suppress colony formation; the cells remained as single cells or small clusters. Because PGE2 is the major downstream molecule produced by COX2 AH6809, an antagonist to the PGE2 receptor, was employed to investigate the role of PGE2 in the growth-promoting effects of TNF $\alpha$  and IL-1 $\beta$  on the BRMS1 cells. AH6809 (50  $\mu$ M) was added to 3D cultures simultaneously with the cytokines (Fig. 2b). As seen previously, cultures containing TNF $\alpha$  and IL-1 $\beta$  contained about twice as many cells as untreated



**Fig. 2** Indomethacin or the PGE2 receptor inhibitor AH6809 blocked the proliferative effects of TNF $\alpha$  and IL-1 $\beta$  or PGE2 on BRMS1 breast cancer cell in a 3D osteoblast culture. **a** TNF $\alpha$ , IL-1 $\beta$ , and BRMS1 cells were added to the 3D cultures and imaged as previously described. The cyclooxygenase inhibitor, indomethacin, was added at a concentration of 50  $\mu$ M to some of the cultures prior to the addition of the cancer cells. Shown are day 2 and 4 images along with % area

fraction plotted over time. **b** The PGE2 receptor antagonist (50  $\mu$ M), AH6809, was added to co-cultures containing TNF $\alpha$  and IL-1 $\beta$ . Shown are images of day 2 and 4 with area fraction analysis graph. **c** Images and area fraction analysis of co-cultures containing TNF $\alpha$ , IL-1 $\beta$  or 300 nM PGE2; AH6809 was added to some cultures. Scale bar 100 microns. \*\*\* $p$  = 0.001; \*\* $p$  = 0.01 relative to untreated cultures.  $n$  = 3 for (a) and (b);  $n$  = 6 for (c)

cultures. This increase in growth was mitigated by AH6809 (Fig. 2b). As with indomethacin, AH6809 alone did not affect the growth of the BRMS1 cells (data not shown).

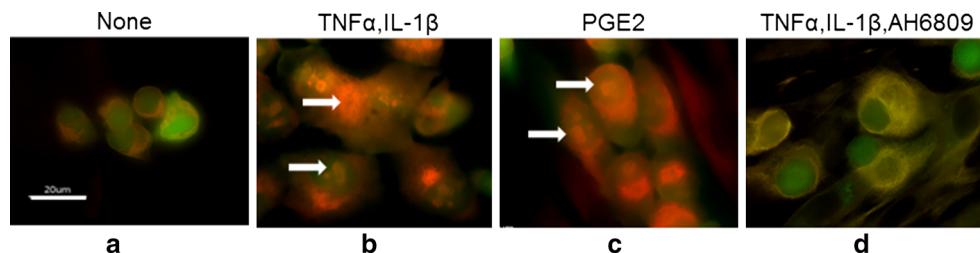
These data suggested that TNF $\alpha$  and IL-1 $\beta$  stimulated BRMS1 growth via PGE2 production. Collected bioreactor culture supernatants were assayed for the presence of PGE2 (Table 1). We found that untreated bioreactors of MC3T3-E1 with or without BRMS1 cells contained approximately 500 pg/mL of PGE2. Addition of TNF $\alpha$  and IL-1 $\beta$  increased the concentration by 60–70 fold to approximately 35 ng/mL. This increase was prevented by addition of NAb to TNF $\alpha$  and IL-1 $\beta$ , and by indomethacin, but not by AH6809.

In order to determine if PGE2 alone was sufficient to cause increased BRMS1 growth, 300nM PGE2 (approximately 35 ng/mL) was added directly to the BRMS1/

**Table 1** Production of PGE2 in bioreactor cultures of MDA-MB-231BRMS1 cells with MC3T3-E1

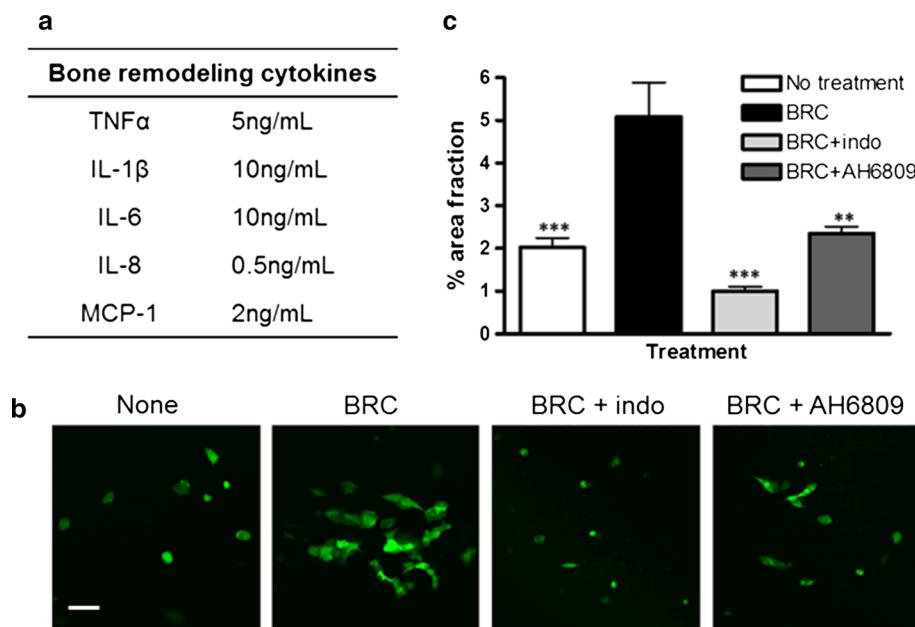
Treatment	PGE2 (pg/mL) $\pm$ SD
None	518 $\pm$ 2
TNF $\alpha$ , IL-1 $\beta$	35,108 $\pm$ 115
TNF $\alpha$ , IL-1 $\beta$ , NAB	368 $\pm$ 171
TNF $\alpha$ , IL-1 $\beta$ , indo	379 $\pm$ 71
TNF $\alpha$ , IL-1 $\beta$ , AH6809	32,450 $\pm$ 2963
Untreated MC3T3-E1	522 $\pm$ 10

3D bone mimetic culture. PGE2 alone brought about a significant increase in BRMS1 cell proliferation (Fig. 2c). The threefold increase was similar to that seen with TNF $\alpha$



**Fig. 3** Nuclear localization of the proliferation marker, Ki67, was increased in co-cultured BRMS1 cells exposed to TNF $\alpha$  and IL-1 $\beta$  or PGE2. Co-cultures were assembled and treated as described in the “methods” section. After 4 days, cultures were disassembled; segments of the culture membrane were fixed and stained by fluorescent immunocytochemistry for the presence of Ki67.

Percentage of cells positive for Ki67 nuclear staining was determined from 50 cells per treatment group. Shown are images from **a** untreated culture (2 %) and cultures treated with **b** TNF $\alpha$  and IL-1 $\beta$  (28 %), **c** PGE2 (24 %) or **d** TNF $\alpha$ , IL-1 $\beta$  and PGE2 receptor inhibitor AH6809 (0 %). Positive nuclear staining is indicated by arrows. Scale bar 20 microns



**Fig. 4** Bone remodeling cytokines (BRC) induced proliferation of dormant MCF-7 breast cancer cells grown in a 3D osteoblast culture. Normal human osteoblasts were grown in the bioreactor for 1 month. The weakly metastatic breast cancer cell line, MCF-7, was added to the growth chamber along with 10 ng/mL bFGF to induce a dormant state. **a** Bone remodeling cytokines TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and MCP-1 were added to the growth chamber at the concentrations

indicated. The COX inhibitor, indomethacin, and the PGE2 receptor antagonist, AH6809, were added to some of the cultures at 50  $\mu$ M final concentration. **b** Live cell images were captured by confocal microscopy; shown are representative images of day 4 cultures. **c** Area fraction analysis of live cell images on day 4. Scale bar 100 microns. n = 6. \*\*\*p = 0.001; \*\*p = 0.01 relative to treatment with cytokines

and IL-1 $\beta$  (3.7 fold). The cells also formed colonies similar to those seen in the presence of TNF- $\alpha$  and IL-1 $\beta$ . This increase in cell proliferation, colony formation and shape was prevented when the PGE2 receptor antagonist, AH6809, was present in the culture mix.

In order to verify the proliferative state of the BRMS1 cells under the various conditions, immunocytochemical detection of Ki67 was carried out on the co-cultured cells (Fig. 3). In untreated cultures, there was a minimal (2 %) number of BRMS1 cells with nuclear localization of Ki67 (Fig. 3a). Conversely, in cultures containing TNF $\alpha$  and IL-1 $\beta$  (Fig. 3b) or PGE2 (Fig. 3c) Ki67 was localized in the

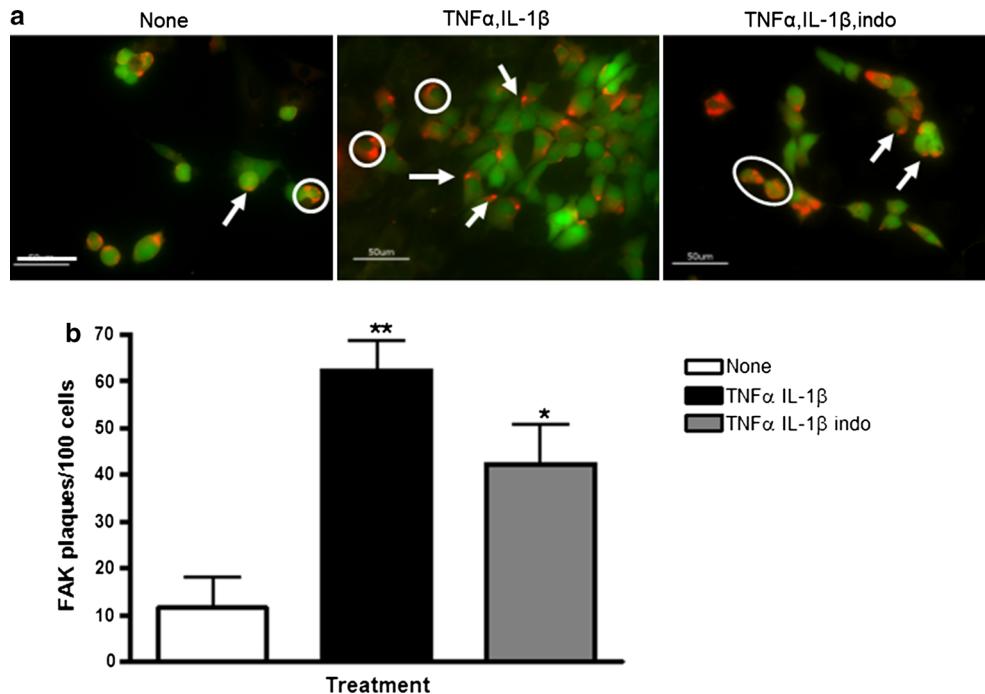
nucleus of 28 % and 24 % of the cells, respectively. AH6809 prevented Ki67 nuclear localization (0 %) in the presence of TNF $\alpha$  and IL-1 $\beta$  (Fig. 3d).

#### Bone remodeling cytokines stimulated proliferation of dormant cells in the MCF-7 model

MCF-7 is a human, ER+, breast cancer cell line, used to model dormancy in the presence of fibroblast growth factor, FGF [41]. In our 3D bone model, these cells entered a dormant state when co-cultured with either normal human osteoblasts (NHOst) or MC3T3-E1 with 10 ng/mL FGF (see Fig. 4b and

**Fig. 5** Focal adhesion plaque formation was increased in co-cultured BRMS1 cells exposed to TNF $\alpha$  and IL-1 $\beta$ . 3D co-cultures of osteoblasts and cancer cells were assembled and treated as described in “Materials and methods” section. After 4 days in culture, bioreactors were disassembled and portions of the membrane stained for phospho-FAK.

**a** Cells displaying cytoplasmic distribution of FAK are circled; focal adhesion plaques are indicated by arrows. Scale bar 50 microns. **b** Graph of focal adhesion plaques seen per 100 cells in three fields of view ( $n = 3$ ). \*\* $p = 0.01$ ; \* $p = 0.05$  relative to untreated cultures



data not shown). A panel of human bone remodeling cytokines (Fig. 4a) was tested in the MCF-7/NHOst system to determine if proliferation was catalyzed as in the MC3T3-E1/BRMS system. As predicted, the MCF-7 cells broke dormancy and proliferated in the presence of the cytokines (Fig. 4b). While untreated cells remained in small clumps, cells treated with cytokines expanded into larger colonies. As seen with the BRMS1 cells, both indomethacin and AH6809 prevented this change in proliferation and colony formation (Fig. 4b, c).

#### Focal adhesion kinase plaque formation was upregulated by TNF $\alpha$ /IL- $\beta$ in BRMS1 dormancy model

One of the reported effects of PGE2 on cancer cells is an increase in focal adhesion kinase (FAK) plaque formation [33]. Therefore, we examined FAK plaque formation in BRMS1 cells cultured on MC3T3-E1 osteoblasts in the 3D chamber with addition of TNF $\alpha$  and IL-1 $\beta$  (Fig. 5). The increase in plaque formation in the presence of cytokines (Fig. 5a, center) was clearly seen when compared with cultures with no additions (Fig. 5a, right) or with cytokines plus indomethacin (Fig. 5a, left). Furthermore, the cytokine-treated cells displayed a distinctly different morphology than the untreated or indomethacin treated cells. Quantification of the FAK plaques revealed an approximately sixfold increase in plaques in the cells treated with TNF $\alpha$  and IL-1 $\beta$  over those without cytokines. Indomethacin reduced plaque formation, but failed to restore it to the untreated level (Fig. 5b).

#### Discussion

In summary, we have used a 3D model of a bone mimic to investigate possible mechanisms for breast cancer cell dormancy and recurrence in the bone. Two cancer cell dormancy models, MDA-MB-231BRMS1 and MCF-7, were utilized. In previous studies in mice, BRMS1 formed primary tumors and trafficked to the bone when introduced by intracardiac injection where they appeared to remain dormant [29, 42]. Additionally, these cells grew poorly on an 3D osteoblastic matrix [27]. However, addition of a set of cytokines associated with bone repair and remodeling, specifically TNF $\alpha$  and IL-1 $\beta$ , induced the BRMS1 cells to proliferate. In the presence of TNF $\alpha$  and IL- $\beta$ , the co-cultures were discovered to produce large amounts of PGE2. Inhibition of PGE2 production with indomethacin or blocking its receptor reversed the cytokine effect on BRMS1 proliferation. Addition of exogenous PGE2 also caused the cells to break dormancy and proliferate. Similar findings were seen with a non metastatic cell line, MCF-7 grown on human osteoblasts in the 3D culture system. These data suggest that PGE2 is a key effector in the breast cancer cell “dormant-to-proliferative” transition in the bone microenvironment.

Prostaglandin E2, the major product of activation of COX-2, plays an important role in normal bone physiology as well as in cancer and bone metastasis (review [43]). In the normal bone, PGE2 is the major prostaglandin and is a strong stimulator of both bone resorption and bone production. It is also elevated under conditions of inflammation

associated with diseases such as rheumatoid arthritis. Moreover, high levels of COX-2 and PGE2 are indicators of poor prognosis for breast cancer patients [44]. COX-2 is reported to be expressed in 40 % of human invasive breast cancers [45].

It has been known for many years that malignant breast cancer cells produce high levels of prostaglandins [46]. Results of studies with mice have provided strong evidence that COX-2 expression is important in bone metastasis. In one study, it was found that breast cancer cells recovered from metastases in the bone produced more prostaglandins than the cell line initially injected into the mice; COX-2 overexpressing breast cancer cells enhanced bone metastases; and finally, an inhibitor of COX-2 reduced the formation of bone metastases [45].

Our data support the premise that BRMS1 cells remain dormant, in part, because they do not produce COX-2 and therefore, PGE2. In fact, Cicek et al. reported that the BRMS1 protein inhibits activation of NF- $\kappa$ B and expression of COX-2 [47]. In a comparison study of MDA-MB-231 and MDA-MB-231BRMS1 cells, they found that BRMS1 cells showed reduced expression of both constitutively produced and TNF $\alpha$ -induced NF- $\kappa$ B. Since COX-2 expression is an indicator of NF- $\kappa$ B activity and PGE2 production dependent on COX-2, we can infer that PGE2 likely plays a major role in the BRMS1 dormancy model. The source of the elevated PGE2 levels seen in the bioreactor is likely the osteoblasts rather than the cancer cells. MC3T3-E1 cultured alone or together with BRMS1 in the bioreactor without additional cytokines produce approximately the same levels of PGE2 (Table 1). It has been known for some time that MC3T3-E1 produce PGE2 when stimulated by TNF $\alpha$  or IL-1 $\beta$  [31, 32]. Although, TNF $\alpha$  and IL-1 $\beta$  bind to different receptors, they are known mediators of bone resorption [48]. Interestingly, it also has been reported that MCF-7 cells do not express COX-2 [49] which may, in part, explain their non metastatic potential.

Clinical dormancy of breast cancer is well known and documented. However, this phenomenon is notoriously difficult to study. From an experimental approach, dormancy is often considered as cellular, angiogenic, or immune related [50]. Cellular refers to mechanisms that keep cells in a quiescent state. Angiogenic dormancy suggests the lack of vascularization limits tumor mass. Immune mediated dormancy implies that host immunosurveillance normally keeps the tumor cells in check. In reality, all of these mechanisms and others are likely involved. In this study, we focused on cellular dormancy as dictated by the local microenvironment.

Many environmental factors can influence metastasis including those produced during resection of the primary tumor [19]. Stress of various kinds can change the environmental milieu of hormones and cytokines. Under

normal conditions, hematopoietic stem cells, HSC, reside in the bone in a dormant state. However, it is known that normal cell turnover as well as injury and stress can activate these cells [51]. There is evidence that metastasized cancer cells occupy the same niche as HSC in the bone [52]. Perhaps the metastases respond to the same stress signals.

Focal adhesion kinase is a PTK2 protein tyrosine kinase, encoded by the *PTK2* gene. This kinase concentrates in focal adhesions that form as cells attach to the extracellular matrix. When FAK is reduced, breast cancer cells are less metastatic due to decreased mobility [53]. FAK foci are affected by integrin activation, growth factor stimulation, and action of mitogenic neuropeptides. PGE2 has been reported to increase focal adhesion kinase in breast cancer [33] a phenomenon observed in this study when the BRMS1 were treated with the bone remodeling cytokines TNF $\alpha$  and IL-1 $\beta$ .

In the 3D model system reported in this study consisting of MDA-MB-231BRMS1 cells co-cultured with a well differentiated osteoblast matrix, BRMS1 behaved as dormant cells; i.e. they did not proliferate. However, addition of cytokines, TNF $\alpha$  and IL-1 $\beta$ , that stimulate production of PGE2, led to a break in dormancy resulting in cell proliferation as evidenced by nuclear localization of Ki67. Addition of PGE2 directly to the cultures had the same effect. Moreover, inhibition of COX or the PGE2 receptor prevented cell proliferation. A downstream effect of increased PGE2 activity is a corresponding increase in focal adhesion kinase plaque formation resulting in increased cell spreading and matrix adhesion. Although PGE2 is required for normal bone homeostasis, elevated levels in the bone microenvironment may trigger dormant breast cancer cells to proliferate. The results of this study provide a plausible explanation for the emergence of latent metastases following skeletal trauma. It is under conditions such as bone repair in which there is an osteoblast inflammatory response, that large amounts of PGE2 are produced locally. These findings provide evidence for dormant cells growth following exposure to specific inflammatory cytokines that elevate local concentrations of PGE2.

In a 2009 review, Naumov et al. [6] discuss clinical dormancy and possible mechanisms. They point out that many individuals carry microscopic tumors that remain dormant for life. A summary of autopsy studies of individuals who died of trauma indicated that as many as 39 % of the women over 39 years of age harbored microscopic breast cancer. Similar incidents were seen with prostate and thyroid cancers. The authors present case studies where trauma was linked to the rapid appearance of lymphomas which had apparently been occult for years. As we pointed out in the introduction, there are other case studies of individuals in which metastasis occurs many years after

removal of the primary tumor and following trauma or disease of the immune system (e.g. [17]). In summary, there is compelling clinical evidence that cancer cell dormancy exists and that bone trauma may trigger the proliferation of the disseminated cells. However, this clinical phenomenon is difficult to study and valid mouse models are not presently available [54]. The 3D culture system described herein offers an *in vitro* approach to begin to dissect some of the mechanisms related to dormancy with the possibility of creating an animal model in the future.

**Acknowledgments** This work was supported by a pilot grant from METAvivor and by the U.S. Army Medical and Materiel Command Breast Cancer Idea Program, Grant W81WH-1s2-1-0127. We thank Dr. K. Sandeep Prabhu for thoughtful discussion.

**Conflict of interest** The authors declare that they have no conflict of interest.

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