Facilitating Role of Preprotachykinin-I Gene in the Integration of Breast Cancer Cells within the Stromal Compartment of the Bone Marrow: A Model of Early Cancer Progression

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ABSTRACT

Despite early detection of breast cancer, patients’ survival may be compromised if the breast cancer cells (BCCs) enter the bone marrow (BM). It is highly probable that BCCs enter the BM long before clinical detection. An in vitro coculture model with BM stroma and BCCs (cell lines; primary cells from stage III BC, n = 7, and stage M0, n = 3) mimicked early entry of BCCs into the BM. In coculture, BCCs exhibit contact inhibition and do not require otherwise needed growth supplements. Stromal growth rate was increased 2-fold in coculture. The inclusion of BCCs in stromal support of long-term culture-initiating cell assay frequencies show no difference (38 ± 3 versus 36 ± 6). Nontumorigenic breast cells (patients and cell lines) did not survive in coculture, suggesting that the model could select for malignant population in surgical breast tissues. Cocultures were able to select cells with 73% cloning efficiency, mimicked early entry of BCCs into the BM (13, 14). Thus, if loss or mutation of BRAC1 is involved in the BM (13, 14), it is not surprising that autologous BM transplantation would be disappointing outcome of autologous BM transplantation in BC patients (14) is compounded by CD34 expression on other cancer cells that also show preference for the BM (10, 11). Perhaps, BCCs could also express CD34, a marker that is used clinically to select hematopoietic cells for BM transplantation. Thus, if BCCs do express CD34, it is not surprising that autologous BM transplantation would be compromised. Without the option of hematopoietic repopulation, protection of the resident hematopoietic stem cells will limit the doses of chemotherapeutic agents. Limited cancer treatment would provide an advantage for BCCs in the BM. Given these arguments, it is important to note that cancer treatments with BM stem cell replacement could be improved if the mechanisms of early entry and survival of BCCs in the BM were understood.

INTRODUCTION

Bone marrow (BM) metastasis of breast cancer cells (BCCs) correlates with poor prognosis (1, 2). Many studies have addressed the biology of BC at a time when the tumor burden is evident. At this time, there could be bone invasion, which makes targeting of the cancer cells difficult. Markers to detect BCCs in the BM during the early stages of diagnosis have been identified (3). However, BC studies have not addressed the period when the frequencies of BCCs in the BM might be at a single cell level and perhaps lower than the frequency of BM stem cells. We hypothesize that BCCs may enter the BM microenvironment at a time long before clinical detection. In the BM, the BCCs find a microenvironment that is conducive to survival while evading treatments. On the basis of this line of thought, it is not surprising that high-dose chemotherapy with hematopoietic stem cell transplantation has failed (4), followed by the closure of five similar clinical trials by the National Cancer Institutes. Such studies are particularly important because of the limitations, e.g., false negatives in BC screening by mammogram and self-examination (5). Despite seemingly curative therapy, cancer could resurge in the BM even 10 years after cancer remission (6). Furthermore, BCCs could metastasize from the BM to other tertiary sites. Although it is established that BCCs show preference for the BM (7, 8), the following remain undefined: the signature of the cancer cells that evade the innate immune system and enter the BM at a period before clinical detection or early during cancer development; the area in the BM where the BCCs are located; and the mechanisms that allow the BCC to survive within the BM microenvironment.

The BM limits the dose of current cancer treatments, mostly because of potential toxicity to the finite number of lymphohematopoietic stem cells that replenish the adult immune system (9). The disappointing outcome of autologous BM transplantation in BC patients (4) is compounded by CD34 expression on other cancer cells that also show preference for the BM (10, 11). Perhaps, BCCs could also express CD34, a marker that is used clinically to select hematopoietic cells for BM transplantation. Thus, if BCCs do express CD34, it is not surprising that autologous BM transplantation would be compromised. Without the option of hematopoietic repopulation, protection of the resident hematopoietic stem cells will limit the doses of chemotherapeutic agents. Limited cancer treatment would provide an advantage for BCCs in the BM. Given these arguments, it is important to note that cancer treatments with BM stem cell replacement could be improved if the mechanisms of early entry and survival of BCCs in the BM were understood.

BRCA1 tumor suppressor gene is associated with familial link of BC and ovarian cancer (12). Interestingly, although BRCA1 is linked to both BC and ovarian cancer, only BC preferentially metastasize to the BM (13, 14). Thus, if loss or mutation of BRCA1 is involved in BM metastasis to the BM, the major mechanism might involve other genes. This study provides evidence that preprotachykinin-I (PPT-I), a gene that is conserved throughout evolution, is central to early metastasis of BCCs to the BM. Enhanced and constitutive expression of PPT-I are related to the development of endocrine and endocrine-related cancers, including BC, most of which preferentially metastasize to the BM (15, 16).

PPT-I is a single copy gene with seven exons (16). PPT-I produces multiple peptides through alternate splicing and posttranslational modification (16). Substance P (SP) is the most studied and the major peptide produced by the PPT-I gene (16). In most immune and BM-derived cells, PPT-I expression requires cell stimulation, e.g., by cytokines (16). The association of the PPT-I gene to BC and BM functions (15) suggests that PPT-I may reveal clues into the mechanism of BCCs in the BM. PPT-I peptides interact with two natural G-protein-coupled receptors, neurokinin (NK)-1 and NK-2. In normal BM stroma, NK-1 and NK-2 regulate the expression of each other and also mediate opposing functions (16). In BCCs, however, NK-1 and NK-2 mediate cell proliferation through autocrine stimulation (15).

Biological functions of PPT-I peptides include those that are ame-
able to tumorigenesis. Thus, the PPT-I gene can be central to other molecules, e.g., cytokines (17–19), that induce changes in BM cells to facilitate BCCs to become part of the BM microenvironment. In this study, we use different experimental tools to study the roles of PPT-I in early seeding of BCCs to the BM. The model used a coculture system of BCCs (cell lines and primary cells) and primary BM stroma (20).

MATERIALS AND METHODS

Reagents and Antibodies. Protease inhibitor mixture, PKH26, hydrocor-tisone, glutamine, and α-MEM tissue culture media were purchased from Sigma (St. Louis, MO). The method of handling both forms of SP was described previously (21). Phycoerythrin (PE)-cytokeratin monoclonal anti-body (mAb), PE-rat antimonoclonal κ, and PE-CD14 mAb were purchased from BD Bioscience (San Jose, CA). Proyl-4-hydroxylase mAb was purchased from Dako (Glostrup, Denmark). Dynabead-Epithelial and antiﬁbroblasts were purchased from Dynal Biotech (Oslo, Norway) and Miltenyi Biotec (Auburn, CA), respectively. FCS and horse sera were purchased from Hyclone Labora-
tories (Logan, UT).

Mice. Female athymic nude mice (BALB/c background, 6–8 weeks) were obtained from The Jackson Laboratory. Mice were housed in laminar flow hood at an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. BCCs, cell lines, or primary cells (refer to selection of malignant cells from surgical samples, below) were labeled with PKH26 as described previously (22). Immediately after labeling, cells were analyzed by immunofluorescence to determine the efficiencies of PKH26 incorporation. The results show >90% of the cells were positive for PKH26.

Mice were euthanized by the method approved by the Institutional Animal Care and Use Committee of New Jersey Medical School. Femurs were flushed once but slowly, with media through a 26-guage needle attached to a 3-ml syringe. These cells were designated as BCCs from cellular areas of the BM. After this, the femurs were opened by longitudinal section and then washed by shaking in PBS (pH 7.4). The wash step was repeated three times with a fresh change of PBS. Sterile blunt end spatulas were then used to scrape cells that were attached to the endosteal region. We generally obtain ~100–150 BCCs from two femurs after scraping. Thus, for each experiment, cells were pooled from five mice. Microscopic immunofluorescence indicated that 50–65% of the cells stained positive for cytokeratin (indicative of the injected BCCs) and 25–40 stained positive for antiﬁbroblasts (indicative of stromal cells). The BCCs were positively stained with antiﬁbroblasts (described below).

Primary Breast Tissue. Breast tissues were obtained from excess tissues taken from patients with stages IIIA or IIB BC. At the time of surgery, patients were not subjected to chemotherapy or radiation. The use of breast tissues followed guidelines of the Institutional Review Board, Newark Campus (Institutional Review Board). Patient 7 was obtained from Cooperative Human Tissue Network, University of Pennsylvania Medical Center (Philadelphia, PA). Table 1 shows the hormone receptor status of patients and the SP levels produced by patients’ cells.

Breast cells (normal and malignant) were retrieved from the breast tissues as follows: (a) flushing with a 1-cm syringe containing culture media; and (b) dislodging with serrated-end forceps. Fibroblasts were depleted from the cell suspension by dual selection process with antiﬁbroblasts microbeads. Flow cytometry with FITC-antiﬁbroblasts and PE-anticytokeratin verified that the cell suspension was devoid of antiﬁbroblasts. Biopsies from three different pa-
tients with stage M0 BC (Table 1) were obtained and also cleared of fibroblasts (as above). Fibroblast-depleted cells from patients with BC are hereafter referred as breast cells because the cells might contain both malignant and normal cells. After cocultures and the anchorage independent cells were selected (below in results), the cells were referred as primary BCCs.

Cell Lines. All cell lines were purchased from American Type Culture Collection (Manassas, VA). The tumorigenic cell lines are as follows: ZR-75-30; BT-474; T47D; MDA-MB-330; DU4475; BT 483; and SK-BR-3. Tumor-
genic cell lines, at 80% confluence in 2 ml of media in 6-well plates, produced SP levels between 34 and 45 pg/ml. The nonmalignant (SP not detected) are as follows: MCF12A; MCF-12F; Hs578St; MCF 10A; and MCF-10 A. 3

Table 1 BC patients (P1–P7) included in the studies

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age (yrs)</th>
<th>Estrogen receptor</th>
<th>Progesterone receptor</th>
<th>c-erbB-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0 (n = 3)</td>
<td>40–55</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P1</td>
<td>73</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P2</td>
<td>60</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P3</td>
<td>64</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P4</td>
<td>82</td>
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<td>P6</td>
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</tr>
<tr>
<td>P7</td>
<td>56</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Telomerase Activity. Telomerase activities from primary BC cells and BC cell lines were studied in duplicates using the Telo TAGGG Telomerase PCR ELISA PLUS kit (Roche Molecular Biochemicals, Indianapolis, IN). Cell lysates from 2 × 10^6 cells were analyzed in PCR reactions for telomerase activity. Absorbance at 450 nm for positive controls ranged between 0.560 and 0.581, and negative controls ranged between 0.010 and 0.023. Positive controls were normalized to 1 and then used as reference for changes in the unknowns.

BM Stromal Cells. BM stroma was established from BM aspirates of healthy individuals as described previously (21). Use of BM aspirates was approved by the Institutional Review Board. BM stroma was cultured in α-MEM containing 12.5% FCS, 12.5% horse sera, 0.1 μM hydrocortisone, 0.1 μM 2-mercaptoethanol, and 1.6 mg glutamine (21). At cell confluence, tryp-
inum-sensitive, adherent cells were passaged at least five times. Stromal cells were CD14 (−) and prolyl 4-hydroxylase (±), as determined by immunofluorescence (21).

Cocultures of BCCs and BM Stromal/Growth Curve. Equivalent numbers of BCCs and BM stroma were cocultured in stromal media with weekly replacement of 50% culture media. At different times, cells were trypsinized and the BCCs were positively selected (twice) with Dynabead conjugated anticytokeratin, as described previously (23). Both cell populations (stroma and BCCs) were counted and the purity (>99% ±) of each verified by flow cytometry with PE-cytokeratin mAb and prolyl 4-hydroxylase mAb-PE-rat antimonoclonal κ IgG for stroma.

Production of SP-Infrared (IR) in BCCs from Patients with Stage M0 BC. After the selection of BCCs from cocultures, cells were resuspended in α-MEM containing 2% FCS. A total of 10^5 cells in 1 ml was added to the wells of 12-well tissue culture plates. After 24 h, cell-free supernatants were collected and then stored at −80°C as aliquots in siliconized tubes until ready to be assayed for SP-IR as described previously (23).

Long-Term Culture-Initiating Cell (LTC-IC) Assays. Limiting dilution method determined the frequencies of LTC-IC in cultures with supporting layers of stroma or cocultures of stroma-BCC in 96-well plates (24). At confluence, the supporting cells were γ-irradiated with 150 Gy. After 16 h, media were replaced with 150 μl of fresh media containing 20 replicates of BM mononuclear cells at 10^5, 5 × 10^4, 10^3, and 10^2/well. There were weekly replacements of 50% culture media. At weeks 6, 8, and 12, the adherent and nonadherent cells from each well were combined in 100 μl of assay media and then studied in clonogenic cultures for the following BM progenitors as previously described (23): erythroid; granulocytic-monocytic; and mixed pro-
genitors. A positive well consisted of more than or equal to one colony. The frequencies of LTC-IC were calculated from the proportion of negative wells and the method of maximum likelihood calculated as described previously (24). The data were analyzed with L-Calc software (Stem Cell Technologies, Vancouver, British Columbia, Canada).

Table 2 Stable Suppression of PPT-I in BCCs. pPMSKH1 was constructed in pBluescript SK+ backbone as described previously (25). The H1 promoter (220 bp) was amplified from pMBH1 (GenBank accession no. X16612), kindly provided by Dr. Sidney Altman (Yale University, New Haven, CT). DNA sequence spanning +145/+315 was amplified by PCR and then inserted in EcoR1/BglII sites of pBluescript. PPT-I-specific small interfering RNA was

8 Internet address: http://www.jax.org.
inserted in BglII/HindIII sites. The BglII site in the gene-specific sequence was modified and was therefore used as an indicator of DNA insertion (see below).

The following is the gene-specific sequence (64 bp) with the inclusion of the loop-forming sequence: 5'gatccc (modified BglII) tttgagcatcttctgcaga (sense, GenBank accession no. NM013997; HindIII) tttcaagaga (loop structure with BglII site) tctgcagaagatgctcaaa (antisense) tttttggaaa (HindIII)-3'.

Control inserts were constructed with single bp changes in three different constructs, hereafter referred as mutant pPMSKH1-PPT-I (Fig. 1). Double-stranded DNA was prepared by annealing 50 µg/ml of each strand at 95°C for 4 min, 10 min at 70°C, and slow cooling at 4°C in buffer with 100 mM K2H2O2, 30 mM HEPES-KOH, and 2 mM MgC2H3O2. Verification of inserts was done by EcoRI/HindIII digestion (284 bp) and DNA sequencing.

BCCs at 60% confluence were transfected with pPMSKH1 or pPMSKH1-PPT-I using Superfect (Qiagen, Valencia, CA). At confluence, culture media were screened for SP-IR. Wells with 20 pg/ml SP-IR were seeded at 10–20 cells/well in 12-well plates. Wells with undetectable SP-IR were then selected by limiting dilution at 0.5–1 cell/well. Clones were assayed as PPT-I (−) if SP-IR and β-PPT-I were undetectable. Reverse transcription-PCR for PPT-I mRNA and SP-IR were described previously (15).

Overexpression of PPT-I in Nontumorigenic Breast Cells. PPT-I was overexpressed in pCEP4-PPT-I (cytomegalovirus promoter) or pREP10/pREP4 (Rous sarcoma virus promoters). All vectors were purchased from Invitrogen (Carlsbad, CA). The coding region of PPT-I spans +1/+454 (GenBank accession no. X54469) and was inserted in both orientations with HindIII and BamHI linkers in the PCR primers.

Clonogenic Assays. Clonogenic assays were performed as described previously (15). Briefly, cells were resuspended in 1.2% methylcellulose containing the media appropriate for the particular BCCs at 105 cell/ml. One ml of cell suspension was added to 35-mm suspension dishes, and the cultures were incubated for 1 week at 37°C. Colonies with ≥25 cells were counted.

Statistical Analysis. Data were analyzed using ANOVA and Tukey-Kramer multiple comparisons test. P < 0.05 was considered significant.

RESULTS

Coculture of BCCs (Cell Lines) and BM Stroma. A coculture method was developed to mimic a model that represents early metastasis of BCCs to the BM. The model assumes that the BCCs will be located in a region with BM stromal cells. Because the BM has low frequencies of stromal cells and the study hypothesizes that few BCCs enter the BM before clinical detection, cultures were initiated with 100 stromal cells/25-cm2 flasks and equivalent numbers of BCCs. The cocultures did not require growth supplements compared with BCCs cultures alone. At cell confluence, both the stroma and BCCs stop dividing and remained viable as determined by trypan blue exclusion. Weekly change of 50% culture media maintained the cocultures as monolayers without the formation of foci up to 4 months. Fig. 2A shows three of seven stroma-BCC (cell lines) and representative BM stroma-BCC cocultures. A, cocultures were initiated with 100 cells from seven different breast cancer (BC) cell lines and bone marrow (BM) stroma. Representative cocultures for three BC cell lines are shown at 2 months after cell confluence. Stromal culture is shown at top right panel. B, stromal cells were selected at different times during cocultures and the data for seven cell lines presented as cell counts versus confluence. Each experimental point (mean ± SD) represents 21 studies because each cell line was assayed with three different BM donors. *, P < 0.05 versus cultures with BM stroma alone. C, growth of BCCs were compared while in cocultures with BM stroma and as separate cultures. *, P < 0.05 versus BCCs in coculture.
stromal culture (top/left panel). The coculture pictures were taken at 2 months after cell confluence. Each cell line was studied with at least three different BM donors. Unlike contact inhibition in cocultures, when BCCs were cultured alone, the cells form foci at confluence and undergo cell death by 2 months in culture (data not shown).

**Growth Curves of Stromal Cells in Cocultures with BCCs (Cell Lines).** We observed that cocultures of BCCs and BM stroma achieved confluence at a faster rate when compared with each cell culture alone. At week 4, the cocultures were confluent compared with 50–60% confluence for stroma alone (Fig. 2B). It could be argued that the relatively larger sizes of the BCCs within the cocultures might explain the rapid attainment of cell confluence. To this end, we performed growth curves of the stromal cells in cocultures and as individual cultures. The results showed that the numbers of stromal cells in cocultures were increased 2-fold over the numbers of stromal cells in separate cultures (Fig. 2B).

The cocultures did not contain growth supplement for the BCCs. The growth requirements for BCCs are provided by BM stroma. We therefore established growth curves for BCCs that were in the presence of stromal cells and then compare the growths with BCCs cultured in its regular culture media. The results showed significantly ($P < 0.05$) slower growth of the BCCs in culture compared with the cells cultured alone (Fig. 2C). The data show that the rate of BM stromal cell growth was significantly ($P < 0.05$) enhanced in cultures of stroma-BCCs.

**LTC-IC Cultures with Monolayer Support of Stromal Cells or Stroma-BCC Coculture.** To determine whether the presence of BCCs (cell lines) in the cocultures can affect hematopoiesis, we performed LTC-IC assays in which the needed stromal support were provided by BM stroma or monolayers of stroma-BCC cocultures. LTC-IC culture (top/left panel). The coculture pictures were taken at 2 months after cell confluence. Each cell line was studied with at least three different BM donors. Unlike contact inhibition in cocultures, when BCCs were cultured alone, the cells form foci at confluence and undergo cell death by 2 months in culture (data not shown).

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**PPT-I as a Facilitator of BCC (Cell Lines) Becoming Part of the Stromal Compartment.** The next set of studies determined whether the PPT-I gene has a role in the integration of BCCs as part of the BM microenvironment. Five different BCCs, stably transfected with pPM-SKH1-PPT-I or three mutants (Fig. 1), were used in cocultures with stroma. The results showed that BCCs that have PPT-I suppressed could not form cocultures, whereas cells with mutant vector form cocultures similar to those in Fig. 2A. Fig. 4 shows growth curves of cocultures with BCCs that have PPT-I suppressed, PPT-I (-), or PPT-I expressed, PPT-I (+). We verified that PPT-I was suppressed in cocultures with PPT-I-suppressed BCCs. This was indicated by undetectable β-PPT-I (by reverse transcription-PCR) in cells expanded from 1 cell taken weekly from the cocultures up to 2 months.

**Role of PPT-I in Contact-Independent Growth of BCCs (Cell Lines).** We next determined whether PPT-I expression was important for anchorage-independent growth of BCCs using two approaches: clonogenic assays were performed with BCCs; BCCs with PPT-I suppressed (small interfering RNA); and nontumorigenic cell lines that were genetically engineered to overexpress PPT-I. PPT-I was overexpressed in pREP10, pREP4, or pCEP4 (Fig. 5A) and then stably transfected in four different nontumorigenic cell lines (MCF12A, MCF10, MCF12F, and MCF10F). The readout for PPT-I expression was as follows: SP-IR levels by ELISA (Fig. 5B) and Western blots (Fig. 5C). PPT-I expressing nontumorigenic cell lines showed foci formation with loss of contact inhibition of cell growth (representative cultures shown in Fig. 5D). Similar foci formation was not observed for untransfected cells, cells transfected with vector alone, or vectors containing inserts that are in the opposite/noncoding orientation of PPT-I (data not shown).

Clonogenic assays with BCCs in which PPT-I was suppressed and nontumorigenic cell lines in which PPT-I was overexpressed showed that PPT-I is important for anchorage independent growth. Representative clonogenic assay is shown for T47D and for MCF10 and MCF12A (Fig. 5E). Similarly, PPT-I expression in four different nontumorigenic cell lines show increased growth of the cells in anchorage-dependent growth (Fig. 5F). For each time point studied, the number to total cells/culture was significantly ($P < 0.05$) increased for PPT-I-expressing cells when compared with cells trans-
fected with vector alone (Fig. 5F). The growth rates for nontumorigenic cells transfected with vector alone and unmanipulated nontumorigenic cells were similar and were therefore combined and presented as PPT-I (−) cells, Fig. 5F. The data described in this section showed that PPT-I is important for transformation of nontumorigenic breast cell lines with respect to anchorage-independent growth and loss of growth inhibition during cell-contact. The results also show that PPT-I increased cell proliferation of nontumorigenic breast cell lines in anchorage-dependent studies.

Selection of Primary BCCs in Cocultures with Stromal Cells. Because the studies shown in Fig. 2 used diverse BC cell lines with respect to hormone receptor expression, we next determined if the data from cell lines could be extended to malignant cells from BC patients. Tissues from 7 different patients were depleted of fibroblasts and then added to stromal cultures or cultured alone in stromal media.

In the absence of stroma, the breast cells did not survive. Representative cocultures are shown for 2 patients (Fig. 6A). The top panel represents 3-day cocultures of passage 0. Cells positively selected for cytokeratin from passage 4 of cocultures were studied for cloning efficiencies and anchorage independence in methylcellulose matrix. Representative colonies for patients (P1–P7) are shown in Fig. 6B. The cloning efficiencies for 200 plated cells at different cell passages are shown in Fig. 6C, represented as the mean ± SD of 32 points. ∗, P < 0.05 versus the corresponding point for BCC (PPT-I−).

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patients with stage M0 BC (Table 1). The results show growth pattern similar to Fig. 6. However, the primary BCCs from stage M0 patients exhibit slower growth rates in cocultures when compared with primary BCCs from patients with stage III BC (data not shown).

The data in this section indicate that anchorage-independent BCCs from patients with stages III and M0 BC can be selected and expanded in stromal cocultures.

SP Production in Primary BCCs from Patients with Stages M0 and III BC. Analyses to determine the levels of SP-IR in breast tissues of patients with stage III BC showed that the PPT-I gene was expressed (Table 1). For patients with early BC, namely stage M0, quantitation of SP-IR was limited by the size of the sample. We therefore subcultured the primary BCCs obtained after four passages in coculture in the absence of stroma. After 24 h of initiating the culture, SP levels were measured in the culture supernatants. Parallel studies were performed with five different primary BCCs from patients with stage III BC. The levels of SP produced by primary BCCs from stage III BC patients were 160 ± 21 pg/ml and stage M0 BC, 55 ± 8 pg/ml.

DISCUSSION

The coculture model was initiated with low frequencies of BM stroma and BCCs (cell lines and from patients) so as to simulate pathophysiology. Both the stromal cells and BCCs rapidly proliferate until cell contact when each cell population transits into cell cycle quiescence (Fig. 2). Although BCCs mediate increases in stromal cell growth (Fig. 2B), the growth rates of the BCCs were significantly reduced (Fig. 2C). Additional studies are required to determine whether the changes in BCC growth are because of functional changes induced by the stromal cells. These questions are currently being addressed with animal models of BC.

Genetic engineering of BCCs (cell lines) in which PPT-I is suppressed or nontumorigenic breast cell lines in which PPT-I was

### Table 2: Breast cancer cells (BCCs) in the bone marrow of nude mice

<table>
<thead>
<tr>
<th>Types of cells</th>
<th>Preprotachykinin-I status</th>
<th>Cells injected</th>
<th>PKH 26 (+) Cells in femurs</th>
<th>Cytokeratin (+) Cells in femurs</th>
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<tbody>
<tr>
<td>BCCs (four cell lines)</td>
<td>+</td>
<td>1000</td>
<td>654 ± 24</td>
<td>690 ± 24</td>
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<tr>
<td>BCCs (stage M0, n = 3)</td>
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<td>758 ± 34</td>
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<td>337 ± 18</td>
<td>326 ± 12</td>
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<tr>
<td>BC cell lines</td>
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<td>Undetectable</td>
<td>&lt;10</td>
</tr>
<tr>
<td>BCCs (stage III, n = 4)</td>
<td>-</td>
<td>1000</td>
<td>Undetectable</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Nontumorigenic breast cells (n = 4)</td>
<td>-</td>
<td>1000</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
</tbody>
</table>
overexpressed shows that the PPT-I gene could facilitate the early integration of BCCs into the BM. On the basis of the interactions between BCCs and stromal cells, we extrapolate that at the early phase in the BM, BCCs might show preference for the region of stromal cells.

Unpublished studies argue for the in vitro model as representative for a period when few BCCs enter the BM, perhaps before clinical detection: BCCs form foci when they adhere to stroma in cultures with higher ratio of stromal cells to BCCs. This is in contrast to the present studies, which show that the monolayer structures are retained 4 months after cell confluence. The uniqueness of our model is shown when the current studies are compared with other studies, which use confluent stroma to show differences in adhesion by malignant and nontumorigenic breast cells (26).

Three observations warrant discussion. (a) Growth supplements required by BC cell lines are not needed in cocultures, suggesting that the stromal cells are important for the survival of BCCs in the BM. The growth curve (Fig. 2B) suggests that the stromal cells are responsive to the BCCs. The significance of these findings is part of ongoing research in the laboratory. It is important to note that the BCCs did not hinder hematopoietic activity (Fig. 3). (b) Regardless of the estrogen receptor status, BCCs (cell lines or primary cells) can form monolayers with stromal cells (Figs. 2 and 6; Table 1). Thus, a recent link between the transcriptional regulator, MTA3, and the estrogen status of BCCs might have to be analyzed differently in the BM (27). (c) The fact that the nontumorigenic breast cells could not survive in cocultures with BM stroma establishes a novel selection process to separate malignant cells from nontransformed cells in breast tissues.

The specificity of small interfering RNA with respect to the target genes (28) underscores the relevance of PPT-I in early BCC metastasis to the BM: PPT-I suppression in BCCs and its overexpression in nontumorigenic breast cell lines suggest that PPT-I might be important for early invasion of BCCs to the BM. PPT-I expression in stroma generally requires stimulation (16). However, unpublished studies show that PPT-I expression varies in both BC and stromal cells at different levels of confluence in coculture.

The fact that a neuroendocrine/neurotransmitter-linked gene (PPT-I) is involved in the metastasis of BC to the BM is consistent with the link of functionally related gene, in BM, for small cell lung carcinoma (29). Parts of the descriptive studies in this report set the stage for more mechanistic approaches to understand the early invasion and quiescent nature of BCCs in the BM. Ongoing studies that show changes in groups of genes during different stages of BCC entry and integration among stromal cells are beginning to provide insights into the methods by which the BCCs protect themselves. The ability of BCC-stromal monolayers to support the functions of endogenous hematopoietic stem cells (Fig. 3) suggests that the general concept of epithelial-mesenchymal transition (30) of the BCCs has gone beyond the mere changes that allow the BCCs to leave their site of origin. An important goal of future research is to develop an understanding of stromal transition by BCCs in the BM.

The in vivo studies to discriminate the functions of two populations of cells within the BM, as shown in Fig. 7, A and B, provide insights into possible changes in BCCs in the BM. The growth rates of BCCs that were retrieved from the cellular compartment of the BM and from regions close to the endosteum where stromal cells are located were significantly different. At present, there is no explanation for these changes. However, we propose two explanations for the observations that slow-growing BCCs are located close to the stromal region: (a) BM-derived cells that are located in the vicinity of the stromal region might induce functional changes in the BCCs; and (b) only the relatively quiescent BCCs enter and integrate within the region close to the endosteum. Ongoing studies will use clones of BCCs and in situ staining to identify the phenotype of the cells surrounding the BCCs.

The present study will add and also form the impetus for additional studies to understand how BCCs use the plastic nature of cells in the BM microenvironment to evade the immune surveillance and remain undetectable, perhaps at a single cell level, at low frequencies similar...
to those of the hematopoietic stem cells. It is necessary to understand the mechanisms of BC entry and integration in the BM so to treat and prevent the spread of BC. It is important that proper functions of the BM are maintained for immune competence. Because a finite number of hematopoietic stem cells in the BM are required to maintain the person’s immune system throughout life, protecting the hematopoietic stem cells is an important consideration in cancer treatment and preventive strategies. Early targeting of BCCs in BM will be crucial for proper control of BCCs. Understanding the mechanism of BCCs at different levels of entry in the BM will enable the identification of novel drug targets or perhaps allow changes in current drug targets, ultimately to prevent bone involvement in BC patients.

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Facilitating Role of Preprotachykinin-I Gene in the Integration of Breast Cancer Cells within the Stromal Compartment of the Bone Marrow: A Model of Early Cancer Progression

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