Nuclear Factor-κB Is Central to the Expression of Truncated Neurokinin-1 Receptor in Breast Cancer: Implication for Breast Cancer Cell Quiescence within Bone Marrow Stroma

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Abstract

Breast cancer is a leading cause of mortality among women in the United States. *Tac1* and neurokinin-1 (NK1) are involved in autocrine stimulation of breast cancer cells (BCCs). The single *NK1* gene produces full-length (NK1-FL) and truncated (NK1-Tr) forms. NK1-Tr mediates malignancy in breast cells. We now report a critical role for nuclear factor-κB (NF-κB) in the expression of NK1-Tr, but not NK1-FL, in human BCCs. By Western and Northern blot analyses, NK1-FL and NK1-Tr were coexpressed in BCCs but were undetectable in nontumorigenic cells. Loss of repressive activity within the 5′ flanking region of the *NK1* partly accounts for constitutive expression of *NK1* in BCCs but could not account for the presence of NK1-Tr. Transient transfections with dominant-negative and wild-type IκB show that activation of NF-κB is required for the expression of NK1-Tr. *Tac1* gene was linked to the generation of NK1-Tr because its overexpression in BCCs led to the production of multiple cytokines that can activate NF-κB to mediate NK1-Tr expression. Studies with Tac1 knockdown BCCs and Tac1-expressing nontumorigenic breast cells verified a role for NF-κB in the expression of NK1-Tr. The quiescent phenotype of BCCs on contact with bone marrow stroma was partly explained by decreased NF-κB activation and undetectable NK1-Tr. In summary, this study shows a role for NF-κB in the expression of NK1-Tr in BCCs, which seems to be reversed by bone marrow stromal cells. [Cancer Res 2007;67(4):1653–9]

Introduction

Breast cancer remains a clinical dilemma despite aggressive treatment, education, and early detection campaigns (1). Among women in the United States, breast cancer continues to be the leading cause of cancer-related deaths (1). Metastatic invasion of the bone marrow, a poor prognostic indicator, is unfortunately common and due partly to endocrine-mediated interaction by cancer cells with the supportive bone marrow microenvironment (2). Recent studies have supported the idea of cancer stem cells (CSCs), which may seed the bone marrow in low numbers early in tumor formation and remain dormant only to later expand and contribute to apparent bone invasion (3). In addition, breast cancer cells (BCCs) can form gap junctions with resident bone marrow stroma to attain cell cycle quiescence (4). To assume that BCCs are located within the bone marrow cavity at low frequency before bone invasion is supported by the following: patients who have been diagnosed with breast cancer early and have undergone aggressive treatments show bone metastasis after more than 10 years of cancer remission (2–4).

Breast cancer is linked to the neuroendocrine system as evident by the implication of genes, such as *Tac1* (also referred as preprotachykinin-I; ref. 5). Research on neuroimmune-related molecules in breast cancer development is relatively limited when compared with other molecular studies in breast cancer research. Several lines of basic, translational, and clinical research show that neurotrophic factors and neurotransmitters could be involved in tumorigenesis (6–9). *Tac1* is a single copy gene with seven exons that is conserved by evolution (10). Through alternate splicing and post-translational modification, the *Tac1* gene produces several peptides that belong to the tachykinin family (10). In BCCs, tachykinins interact with neurokinin-1 (NK1) and neurokinin-2 (NK2), both G protein–coupled, seven-transmembrane receptors (11), which leads to autocrine proliferation (5, 9). This is not surprising because NK1 and *Tac1* peptides are also linked to the development of other cancers (7, 12–16).

Constitutive expression of *NK1* and *Tac1* in BCCs highlights their dysregulation because similar expression in normal cells requires cell stimulation (10). Furthermore, coexpression of NK1 and NK2 in BCCs contrasts normal cells, where these two receptors exhibit a yin-yang relationship partly through receptor cross-talk and by specific cytokines (5, 17). *Tac1* is constitutively expressed in BCCs (5, 18). Because its peptides activate NK1, the latter is expected to undergo desensitization (19). BCCs, however, do not show evidence of NK1 desensitization, which seems to be explained by the truncated form of NK1 (NK1-Tr; ref. 19). A 100-amino acid fragment present in the full-length NK1 (NK1-FL) is lacking in NK1-Tr, although the latter can activate G proteins (11). To this end, this study focused on the mechanism by which NK1-Tr is generated from a single *NK1* gene (20). This question is important because NK1-FL mediated slow growth of BCCs and reversed the BCCs to contact inhibition in monolayers (21). In contrast, NK1-Tr enhanced the growth of BCCs, mediated the production of cytokines with growth-promoting properties, and also induced foci formation when expressed in nontumorigenic breast cells (21).

The basis for this study is the multiple cytokines induced by *Tac1* overexpression in BCCs (22). Peptides derived from *Tac1* require the presence of neurokinin receptors for activation (23). Because the nuclear factor-κB (NF-κB) transcription factor is a mediator of cytokine activation, this study shows a role in the generation of NK1-Tr in BCCs. The studies done with BCC lines

Note: This work was done at the Department of Medicine-Hematology/Oncology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey. It is also in partial fulfillment for a Ph.D. thesis of S.H. Ramkissoon.

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were validated with primary BCCs from surgical tissues. A subset of BCCs reverted to a 'dormant' phenotype when they form gap junctions with bone marrow stromal cells (4). This study shows stromal contact correlated with down-regulation of activated NF-κB and undetectable NK1-Tr.

Materials and Methods

Reagents, cytokines, and antibodies. FCS was purchased from HyClone Laboratories (Logan, UT). Rabbit anti-NK1 and Ficoll Hypaque were purchased from Sigma (St. Louis, MO). The following cytokines were purchased from R&D Systems (Minneapolis, MN): interleukin (IL)-1α, IL-1β, IL-6, IL-11, and stem cell factor. Nerve growth factor (NGF) was obtained from Amersham Life Sciences (Arlington Heights, IL). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was purchased from BD PharMingen (San Diego, CA), rabbit anti-p65 was from Santa Cruz Biotechnology (Santa Cruz, CA), and phycoerythrin-cytokeratin monoclonal antibody (mAb) was from BD Biosciences (San Jose, CA). Dynabead-Epithelial and antifibroblast microbeads were purchased from Dynal Biotech (Oslo, Norway). Antifibroblast microbeads and FITC-fibroblasts mAb were purchased from Miltenyi Biotec (Auburn, CA).

Bone marrow stromal cells. Bone marrow stroma was cultured from bone marrow aspirates of healthy individuals as described (5). Use of bone marrow aspirates was approved by the Institutional Review Board of University of Medicine and Dentistry of New Jersey (Newark, NJ). At cell confluence, trypsin-sensitive, adherent cells were passed at least five times and were >99 positive for fibroblasts based on labeling with FITC-antifibroblasts.

Cell lines. All cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and propagated according to ATCC instructions. The following groups of cell lines were used in the studies. (*a*) Tumorigenic: ZR-75-30/ATCC CRL-1504, ascites ductal carcinoma; BT-474/ATCC HTB-20, ductal carcinoma; T47D/ATCC HTB-133, pleural effusion ductal carcinoma; MDA-MB-330, breast carcinoma from pleural effusion; DU4475/ATCC HTB-123, breast carcinoma; BT 483/ATCC HTB-121, ductal carcinoma; and SK-BR-3/ATCC HTB-30, pleural effusion adenocarcinoma. Each of the aforementioned cell lines produces between 35 and 68 pg/mL substance P. (*b*) Nontumorigenic/adherent independent: MDA-MB-330/ATCC HTB-127, showed undetectable substance P. (*c*) Nontumorigenic/adherent dependent: MCF12A/ATCC CR-10782, MCF12F/ATCC CRL-10783, Hs578Bst/ATCC HTB-125, MCF10A/ATCC CRL-10317, and MCF10-2A/ATCC CRL-10781. All cell lines in this group showed undetectable substance P.

Selection and expansion of primary BCCs. Leftover breast tissues from surgical specimens were obtained from Brookdale Hospital (Brooklyn, NY). The tissues were used in this study providing the patients did not receive prior treatment with anticancer agents. The hormone status, stage, and age of the patients (P1, P2, and P3) from which malignant cells were expanded were described previously (21). The studies were approved by the institutional review board of the University of Medicine and Dentistry of New Jersey and Brookdale University Hospital. Expansion of BCCs from surgical tissues was described, and they are hereafter referred to as primary BCCs (24). Nontumorigenic cells do not survive expansion.

Northern blot analysis. Northern blot analyses were done for NK1-FL and NK1-Tr as described. Total RNA was extracted with RNeasy Mini kit (Qiagen, Valencia, CA) and 10 μg were separated by electrophoresis on 1.2% agarose. RNA was transferred to nylon membranes (S & S Nytran, Keene, NH) and then hybridized with cDNA probe and randomly labeled with [α-^{32}P]dATP (3,000 Ci/mmol/L; DuPont/NEN, Boston, MA). Probes were labeled with the Prime-It II Random Primer kit (Stratagene, La Jolla, CA). Bands were normalized by stripping and reprobing with cDNA for 18S rRNA. Membranes were placed in phosphoimager cassettes (Molecular Dynamics, Sunnyvale, CA) and then scanned after 24 h with the Typhoon phosphoimager (Molecular Dynamics). NK1 cDNA probe spanned the coding sequence of NK1-FL (accession number M76675; 1.3 kb). To verify that the lower bands were NK1-Tr, we reprobed the membranes with a cDNA probe that spanned the deleted portion (~300 bp) of NK1-Tr (19, 21). The 300-bp DNA probe was prepared by digesting NK1-FL with *Alu*I and then gel purifying the band equivalent to 300 bp.

Cell membrane extraction and Western blot. Cell membrane extracts were prepared as described (21). Briefly, 2×10^6 cells were placed in a siliconized tube, washed with 1× PBS (pH 7.4), and then incubated at room temperature for 15 min with 400 μL of 1× lysis buffer (Promega, Madison, WI). During the incubation period, the tubes were placed on a tabletop rocker. After incubation, the tubes were centrifuged at 10,000 × *g* at 4°C for 15 min. Supernatants were stored at −80°C in siliconized microcentrifuge tubes, and the membrane-containing pellets were resuspended in 300 μL of 1× PBS. Pellets were vortexed and then analyzed for total proteins. Membrane extracts (20 μg proteins) were analyzed by Western blots for NK1 as described (21). Nuclear extracts were studied for p65. Samples were electrophoresed on 12% SDS-PAGE (Bio-Rad Laboratories, Hercules, CA) and then transferred to Immobilon-P membranes (Millipore, Bedford, MA). Transfer membranes were incubated overnight with anti-NK1 (1:10,000) at 4°C. After this, membranes were incubated for 2 h at room temperature with HRP-conjugated goat anti-rabbit IgG and diluted at 1:10,000. HRP was detected by chemiluminescence. The molecular weight of unknowns was compared with Kaleidoscope Prestained Standards (Bio-Rad Laboratories). The expected molecular weight of NK1-FL and NK1-Tr is 46 and 36 kDa, respectively.

Stable suppression of *Tac1* in BCCs. pPMSKH1-Tac1 was described previously (22). BCCs, at 60% confluence, were transfected with pPMSKH1 or pPMSKH1-Tac1 using SuperFect (Qiagen). At 80% confluence, transfection was verified by screening the culture medium for substance P production using ELISA as described (5). Cultures showing <20 pg/mL substance P were subcloned at 10 to 20 cells per well in 12-well plates. At passage 2, wells showing undetectable substance P were subcloned by limiting dilution at 0.5 to 1 cell per well. If substance P remains undetectable after 10 passages, the cultures were considered to be stably suppressed for *Tac1* and were cryopreserved.

Overexpression of *Tac1* in nontumorigenic breast cells. *Tac1* expression plasmid, pREP10 with Rous sarcoma virus promoter, was described previously (22). Cells were transfected as described above, and stable transfectants were selected with hygromycin. Hygromycin-resistant clones produced >100 pg/mL of substance P at 80% confluence by ELISA.

Transient transfection and reporter gene assay. pGL3 reporter gene (luciferase) with different fragments of the 5′ flanking region of *NK1* was described previously (17). Cells (~80% confluent) were cotransfected with pGL3-NK1 fragments and pβ-gal-control (0.5 μg each) using SuperFect. At 48 h, cell-free lysates were prepared and quantitated for total protein as described (17). The lysates were quantitated for luciferase and β-galactosidase using the Luciferase Assay System (Promega) and the β-Galactosidase Detection Kit II (BD Bioscience Clontech, Palo Alto, CA), respectively. The ratios of luciferase/β-galactosidase in cells transfected with vector alone ranged between 0.18 and 0.19 and were normalized to 1. Transfections were repeated if β-galactosidase activities were <100 relative luciferase units. Total proteins were determined with a kit purchased from Bio-Rad Laboratories.

Suppression of NF-κB in BCCs. Mercury IκBα dominant-negative vector set was purchased from BD Bioscience Clontech. The studies used pCMV-IκBM (mutant) and pCMV-IκBα (wild-type). pCMV-IκBM prevents phosphorylation of IκB, whereas pCMV-IκBα could allow for the activation of NF-κB. BCCs were transfected with pCMV-IκBM or pCMV-IκBα for 48 h. After this, total RNA was extracted and analyzed by Northern blot analyses for NK1-FL and NK1-Tr as described above. Western blots with nuclear extract–transfected cells with pCMV-IκBM showed no band after 48 h (data not shown), whereas similar studies with extracts from pCMV-IκBα transfectants showed bands consistent for p65 (data not shown). Transfections with pCMV-IκBα were used as positive controls.

Cocultures of BCC or primary cells with bone marrow stroma. Cocultures of BCCs with bone marrow stroma were described previously (24). Briefly, BCCs or primary cells were cocultured with equal numbers of stroma in stromal medium. After 48 h, BCCs were positively selected with anticytokeratin-conjugated Dynabeads (Dynal Biotech), whereas bone marrow stroma remained in the negative fraction. Purity of each cell

subset was determined to be >99% by flow cytometry as described previously (24).

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

Results

Role of cytokines in NK1 induction. We have previously reported an enhancement in cytokine production in nontumorigenic breast cells overexpressed with NK1-Tr (21). In a corollary study, reduced production of cytokines in *Tac1* knockdown BCCs led to undetectable NK1-Tr (21). These reports suggest that the expression of NK1-Tr requires costimulation by multiple cytokines, and any single cytokine with stimulatory effects on NK1 would mediate the expression of only NK1-FL. To test this, we stimulated nontumorigenic MCF12A with cytokines, known to induce NK1, and then determined if one or both NK1 transcripts were expressed (21). The neurotrophic factor NGF served as a positive control (21). Previous studies on NK1 expression were done by quantitative reverse transcription-PCR with primers that detected both NK1-FL and NK1-Tr (5). Northern blot analyses have been previously shown to discern both subtypes (21). Representative of three different experiments, shown in Fig. 1 at 12 h of stimulation (optimal time), shows single bands at the predicted sizes for NK1-FL, whereas there was no detectable band in unstimulated cells (Fig. 1, *left lane*).

Repressor activities within the 5′ flanking regions of NK1 in breast cells. *NK1* is a single copy gene, indicating that the two transcripts cannot be derived from separate genes (19, 21). To begin studies that address the mechanisms by which NK1-Tr is derived, we did molecular studies, aided by reporter gene assays. The reporter gene contained various constructs of the 5′ flanking region of NK1. Five different nontumorigenic breast cells (MCF12A, MCF12F, Hs578Bst, MCF10A, and MCF10-2A) were transfected with pGL3, linked to various upstream NK1 fragments (Fig. 2*A*), and were also described previously (20). Luciferase activities indicated that the degree of 3′ deletions was proportional to luciferase activities (Fig. 2*B*).

NK1 shows tissue specificity in its regulation: breast versus stromal cells. Previous studies reported the minimal promoter of NK1 to be upstream of the transcription start site (Fig. 2*A*: NK1-p1; ref. 20). Because we have not observed NK1-Tr in bone marrow stroma (20), we asked whether the NK1-p1 fragment, which lacks the downstream repressor region, is regulated differently in bone marrow stroma and nontumorigenic breast cells. Transient transfections were done with pGL3-NK1-p1 in the same nontumorigenic breast cell lines studied in Fig. 2*B* (n = 5). Each cell line was repeated thrice (n = 15). The difference in luciferase activity by

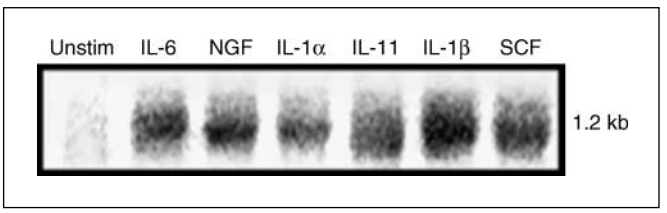

Figure 1. Northern blot for NK1 mRNA in stimulated and unstimulated (*Unstim*) nontumorigenic breast cells. Confluent nontumorigenic breast cells were stimulated with the optimal concentrations of cytokines for 12 h. Following stimulation, total RNA was extracted and NK1 mRNA was evaluated by Northern blot analysis. The cDNA probe spanned NK1 coding region. *SCF,* stem cell factor.

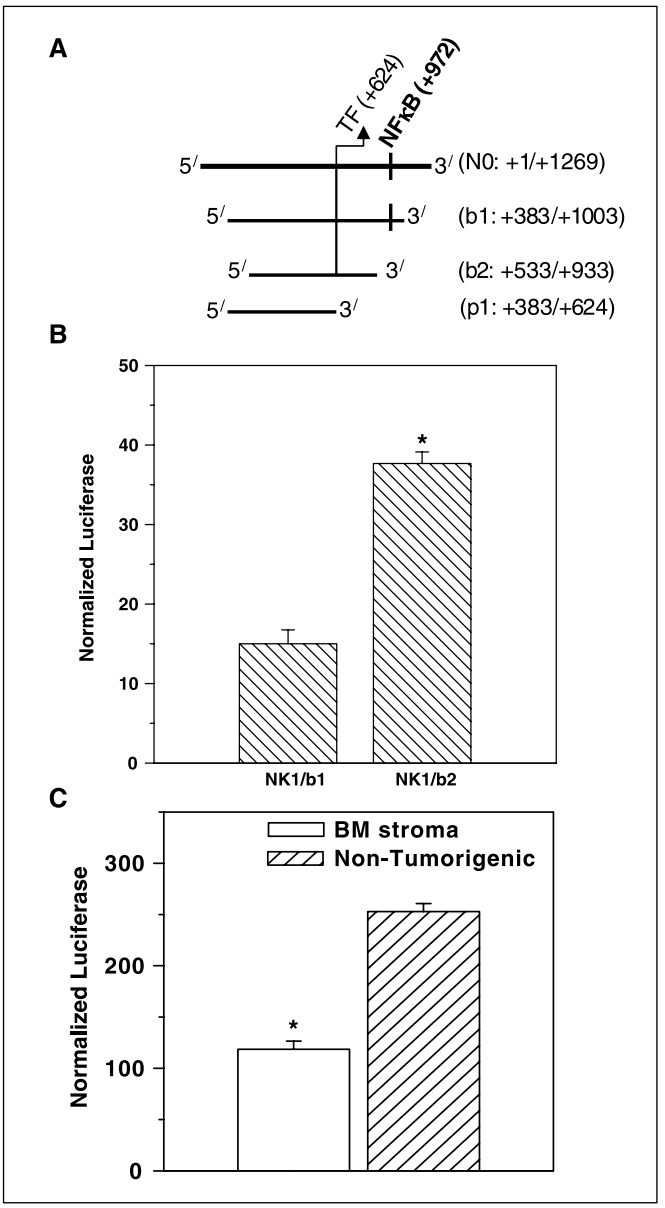

Figure 2. Reporter gene activities for the 5′ flanking region of NK1 in nontumorigenic breast cells. *A,* five different nontumorigenic breast cell lines were transfected with pGL3 containing DNA inserts. *B, Y axis,* luciferase activities for each fragment. *Columns,* mean of normalized luciferase; *bars,* SD. Each cell line was studied in three different experiments. *C,* pGL3 with NK1-p1 insert was transfected into stroma or five different nontumorigenic breast cell lines. *BM,* bone marrow. *Columns,* mean of normalized luciferase activities for 15 experimental points with each cell line assayed in three different experiments; *bars,* SD. Stroma was assayed in three different experiments.

NK1-b1 and NK1-b2 (Fig. 2*B*) can be attributable to deletions in the 5′ or 3′ ends (Fig. 2*A*). However, previous work has shown that the 5′ deletions are not associated with increased reporter expression, and therefore, the difference must be due to 3′ deletions of repressor elements (20).

In nontumorigenic cells, the repressor region-deficient NK1-p1 showed significantly greater activities (P < 0.05; Fig. 2*B* and *C*) when compared with fragments containing the repressive element (NK1-b2 and NK1-b1). Interestingly, NK1-p1 activities in bone marrow stroma were significantly (P < 0.05) increased (Fig. 2*C*) when compared with nontumorigenic cells with constructs

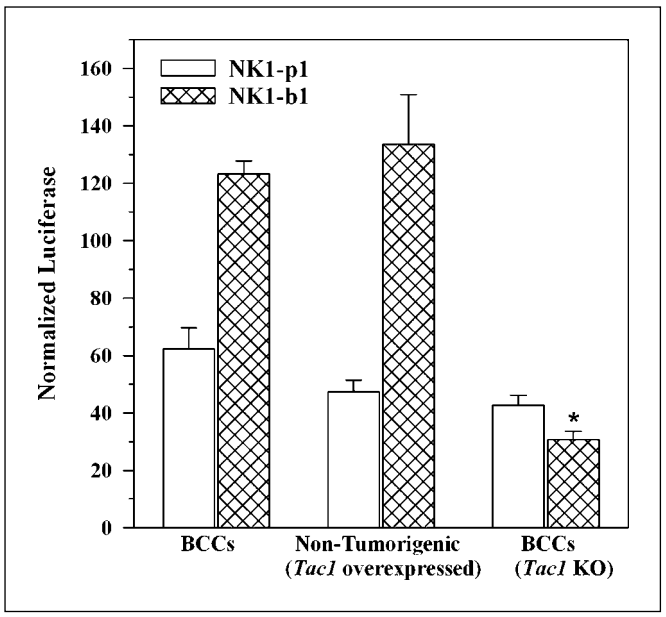

Figure 3. Reporter gene activities of NK1-b1 and NK1-p1. BCCs (*left*), *Tac1*-expressing nontumorigenic cells (*middle*), and *Tac1* knockout (*KO*) BCCs (*right*) were transfected with pGL3-NK1-p1 or pGL3-NK1-b1. After 48 h, luciferase activities were determined. Normalized luciferase as the mean for four different cell lines, each studied in four separate experiments (n = 16).

containing the repressor sequences (Fig. 2*B*). Luciferase activities of NK1-p1 in nontumorigenic breast cells were significantly (P < 0.05) higher than in bone marrow stroma (Fig. 2*C*). The results show that the minimal promoter of NK1 was induced at high levels compared with stromal cells, which represent cells of another tissue where NK1 can be expressed (23, 25).

BCCs show losses in gene repressor functions in NK1 5′ flanking region. Previous studies showed cytokine stimulation reversing the repressor effects within the 5′ flanking region of *NK1* (20). Because BCCs produce several cytokines at high levels, we investigated if, through cytokine production by BCCs (21), the repressor functions with NK1-b1 (Fig. 2*A*) could be reverted in BCCs. This was addressed in three different experiments in which seven different BCCs were transfected with pGL3-NK1-b1 (n = 21). The results showed significant (P < 0.05) increases in luciferase activities (Fig. 3, *left group, hatched column*) compared with similar studies in nontumorigenic cells (Fig. 2*B, left column*), which express baseline cytokines (21).

We next determined if the increase is limited to NK1-b1. To this end, we did reporter gene assay as described above with NK1-p1. The results showed suppression in the BCCs (Fig. 3, *left group, white column*) compared with nontumorigenic cells (Fig. 2*C, left diagonal column*). This difference, combined with the increase by NK1-b1, underscores the importance for reversal of the repressor region in BCCs for NK1 induction.

NK1-b1 activities were enhanced in BCCs. The next set of studies determined roles for cytokines in this increase. Because BCCs induce multiple cytokines (21), a single cytokine might not show a significant reversal (Fig. 1). Thus, to suppress multiple cytokines, the most efficient method is to knock down *Tac1* because this led to global cytokine decrease (22). We therefore transfected Tac1 knockdown BCCs with both NK1-b1 and observed significant (P < 0.05) reduction in luciferase activities (Fig. 3, *right group, hatched column*) compared with untransfected BCCs (Fig. 3, *left group, hatched column*) and nontumorigenic cells where *Tac1* was overexpressed (Fig. 3, *middle group*). The striking similarity by BCC and Tac1 expression nontumorigenic cells, compared with the difference in the Tac1 knockdown cells, might be explained by differences in cytokine production (21).

Role of NF-κB in the expression of NK1-Tr. This section focuses on a role for the transcription factor NF-κB. The premise for this is as follows: cytokines with growth-promoting activities are involved in the activation of NK1 and expression of NF-κB (26–28), deletion of the NF-κB–binding site in the 5′ flanking region led to increased luciferase activity (Fig. 2*B*), and NF-κB has been reported to be critical in NK1 expression (20, 29). We first determined if the NF-κB subunit p65 was present in nuclear extracts from BCCs. Figure 4*A* (*left*) shows representative results of one BCC line and two primary cells. Untransfected MCF12A (nontumorigenic cells) showed no detectable band (Fig. 4*A, middle*). However, MCF12A, stably transfected with pREP10-Tac1, showed a band for p65 (Fig. 4*A, middle*). The presence of nuclear p65 could not be due to the vector because no band was detected for cells transfected with pREP10 alone (Fig. 4*A, middle*). Tac1 knockdown BCCs showed no band for p65 compared with a strong band for BCCs stably transfected with the small interfering RNA (siRNA) vector without insert, pPMSKH1 (Fig. 4*A, right*).

The next set of studies determined cause-effect relationship between NF-κB and NK1-Tr. This question was addressed by

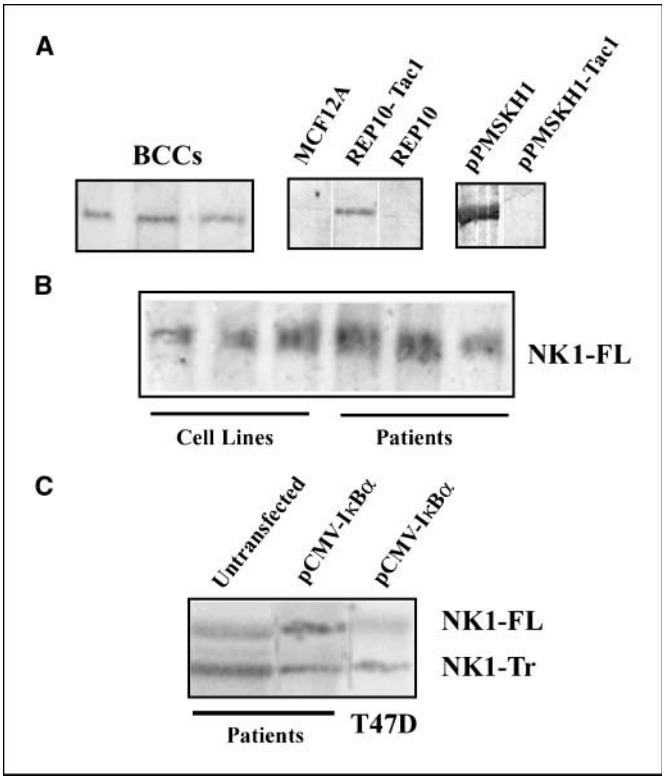

Figure 4. *A,* effect of *Tac1* on NF-κB expression. Representative Western blot for p65 in three different nuclear extracts (*left*), in nontumorigenic cells transfected with *Tac1* (*middle*), and in BCCs with siRNA-mediated suppression of *Tac1* (*right*). *B,* BCCs were transfected with dominant-negative IκB. At day 2, transfectants negative for nuclear p65 by Western blots were analyzed for NK1 mRNA by Northern blot analyses. *C,* representative transfectant with pCMV-IκBα (wild-type) in primary BCCs from one patient (P1), one cell line (T47D), and untransfected cells from P1. After 2 d of transfection, the cells were studied in Northern blot with NK1 cDNA.

transfecting BCCs with dominant-negative IκB (pCMV-IκBM showed) and then studying NK1-FL and NK1-Tr expression by Northern blot analyses. Bands were detected for NK1-FL alone (Fig. 4*B*). Control transfections with wild-type IκB showed nuclear p65 (data not shown). Control studies to show that the results in Fig. 4*B* are specific to the dominant-negative IκB, we transfected BCCs from one patient and one cell line (T47D) with wild-type IκB and then studied the cells for NK1 subtypes by Northern blot analyses. The results show the presence of bands equivalent to the sizes of NK1-FL and NK1-Tr for both the patient and cell line (Fig. 4*C*). The bands show sizes similar to untransfected patient BCCs (Fig. 4*C, left*). In summary, this section describes studies to show a critical role for NF-κB in the expression of NK1-Tr.

Coculture of BCC and bone marrow stroma. Bone marrow involvement by breast cancer is a complex process with proposed mechanisms, including endocrine-mediated and/or chemokine-mediated signaling or, alternatively, primary tumors originating in the bone marrow, which take on characteristic properties of BCCs (4, 30). Once in the bone marrow, BCCs integrate among stromal cells, and through a combination of gap junctions and micro-environmental cues, these invading cells transit to a quiescent state for extended periods, often years (1, 4). To understand how these metastatic cells 'change' on bone marrow integration, we established cocultures using tumorigenic BCCs or primary BCCs with bone marrow stroma. Western blot showed only minimally detectable p65 following coculture (Fig. 5*A*), suggesting a loss of growth-promoting signals following bone marrow integration. Given our previous findings showing the importance of NF-κB in regulating *NK1* (Fig. 4), a decrease in p65 should alter the expression of *NK1*. Western blot for NK1 revealed a loss of NK1-Tr following coculture of a tumorigenic cell line with stroma, although NK1-FL was present (Fig. 5*B*). This finding is consistent with a role for NF-κB in promoting NK1-Tr expression (Fig. 4*B*) but not NK1-FL.

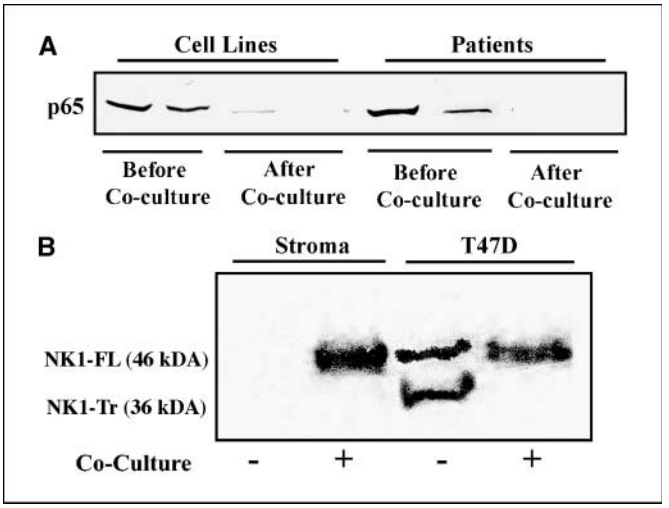

Figure 5. p65 and NK1 expression in cocultures of bone marrow stroma and BCCs. *A,* equal numbers of bone marrow stromal cells were cocultured with tumorigenic cell lines or primary tumor cells for 48 h. Cell subsets were isolated by magnetic bead separation and Western blot done to evaluate p65. *B,* similarly, T47D was cocultured with bone marrow stroma and NK1 expression was determined by Western blot.

Discussion

Despite advances in imaging technology, patient/clinician education, and successful awareness campaigns, breast cancer continues to be a leading cause of death in women (1, 2). Traditionally, it has been proposed that, with early detection of subclinical lesions and appropriate combination treatment, a woman can be 'cured' of breast cancer. However, all too often, apparent curative therapies give way to resurgent disseminated disease despite no obvious primary breast mass (1, 2). Women with early diagnosis breast cancer who opted for aggressive surgical and medical intervention occasionally present again with metastatic breast cancer, presumably from a bone marrow source. CSCs have been proposed as the source of reemerging tumor (3, 31, 32). CSCs are believed to seed the bone marrow at an early period of tumor formation where the cells remain dormant for years only to reemerge following unknown stimuli.

An alternative to the CSC theory is the change in phenotype of BCCs on contact with bone marrow stroma, partly through the generation of gap junction (4). Stromal cells induce a quiescent phenotype in BCCs with the latter adapting nonaggressive properties (4). As such, it is important not only to understand mechanisms leading to tumor formation but also to investigate how these pathways are altered following bone marrow integration. In this study, we show a central role for NF-κB in regulating the expression of NK1-Tr, an oncogenic isoform of NK1, previously shown to be capable of transforming breast cells. Further, we show that, on bone marrow integration, tumor cells lose expression of NK1-Tr but retain NK1-FL. Because the latter has been shown to mediate reduced cell growth compared with NK1-Tr (21), then decreased expression of NK1-Tr suggests that bone marrow microenvironmental stimuli promote a less aggressive or quiescent phenotype. Additional studies are needed to fully appreciate tumor biology within and outside the bone marrow in order to develop targeted therapeutics and improved patient survival.

BCCs (cell lines and primary cells) express both NK1-FL and NK1-Tr (21). The deleted portion of NK1-Tr removes residues that are important for desensitization but retains the ability to signal via protein kinase C. With no definitive explanation for the expression of NK1-FL and NK1-Tr in BCCs, we evaluated the 5′ flanking regions of *NK1* in nontumorigenic cells. The results indicate a region with repressor activity downstream of the transcription start site as evidenced by maximal reporter activity when this region was deleted (Fig. 2), a finding similar to that seen in bone marrow stroma (20). Dysregulation or reversal of this repressor activity is critical because its absence results in abnormally high levels of NK1 transcription. Normal stroma and nontumorigenic breast cells transfected with NK1 minimal promoter constructs lacking the 5′ repressor region both showed elevated reporter activity (Fig. 2*C*).

Growth-promoting cytokines that induce endogenous NK1 have been shown in the hematopoietic system to reverse the downstream repressive effects, whereas growth-suppressive cytokines (e.g., transforming growth factor-β1) could not block this effect (20). We showed up-regulation of NK1 in stroma following stimulation with various single cytokines (20) and therefore investigated if individual cytokine known to be produced by BCCs could exert similar functions in nontumorigenic breast cells. Indeed, we have observed NK1 mRNA consistent with the full-length type but not the truncated form (Fig. 1). This suggests that a single cytokine is insufficient to induce NK1-Tr, thereby underscoring a role for multiple cytokines in the generation of NK1-Tr.

Transfection with NK1-b1 or NK1-p1 in BCCs resulted in increased luciferase activity for NK1-b1 despite the presence of the repressor region (Fig. 3). As *Tac1* is constitutively expressed in BCCs and its peptides are known to induce cytokines capable of regulating NK1 expression, nontumorigenic cells expressing *Tac1* were similarly evaluated. Results were comparable with BCCs and suggested a definitive role for *Tac1* and its peptides in suppressing the repressor activity, 3′ to the transcriptional start site. BCCs lacking *Tac1* and its induced cytokines showed greatly decreased NK1-b1 activity likely due to repressive effects (Fig. 3).

In addition to inducing *NK1* expression, growth-promoting cytokines are also known to regulate NF-κB, and we therefore investigated whether NF-κB activity is relevant to NK1 expression. The reasons for these studies were as follows: (*a*) NF-κB–interacting sites on NK1 promoter are important for its activation (20), (*b*) Tac1 peptides can induce cytokines that are capable of activating NF-κB (29), and (*c*) NF-κB is proposed as a target for cancer (28). NF-κB is constitutively active in many types of cancer, including breast cancer, and therefore any ability of NF-κB to regulate NK1 would be important to understand for future therapeutic potential. Our results suggest that not only is NF-κB expressed in BCCs but also expression is partly dependent on *Tac1* activity. In light of *Tac1* peptides inducing the production of multiple cytokines, it is highly unlikely that the direct signaling is solely responsible for NF-κB activation. It is likely that the cytokines produced by Tac1 peptides indirectly activate NF-κB. In addition, a diverse range of tumors overexpresses NF-κB in the absence of *Tac1* expression, thereby suggesting other mechanisms involved in NF-κB signaling in BCCs (28, 29). In BCCs where NF-κB activity was suppressed by dominant-negative IκB, only NK1-FL transcript could be detected, whereas BCCs with increased NF-κB showed both forms of the NK1 transcript (Fig. 4*B*). Having previously established a role for NK1-Tr in transforming nontumorigenic breast cells (21), it seems that, by driving the transcriptional machinery, overexpression of NF-κB promotes NK1-Tr production and ultimately phenotype transformation. Further studies are needed to evaluate the role of cytokine stimulation, autocrine or reactive, on NF-κB/NK1-Tr–mediated transition of breast cells from normal to dysplastic to carcinoma *in situ*.

Breast cancer preferentially metastasizes to the bone marrow by following homing signals established for hematopoietic stem cells (23, 25). The bone marrow cavity is a unique microenvironment rich in regulatory molecules necessary in maintaining normal hematopoietic and immune functions. As such, breast cancer integration into the bone marrow represents an ideal microenvironment where tumor cells must 'adapt' to survive. If there are a subset of cancer cells that preferentially seed at an early period in the bone marrow, these cells must be aggressive enough to escape the primary tumor site yet adaptable enough to remain dormant within the stromal compartment of the bone marrow. We showed that, on integration into the bone marrow, breast cancer (primary and cell lines) down-regulates NF-κB and shows loss of NK1-Tr expression (Fig. 5).

The mechanisms governing early entry of BCCs into the bone marrow are unclear, but BCCs do respond chemotactically to high levels of stromal-derived growth factor-1α (SDF-1α) produced by bone marrow stromal cells (4). Circulating tumor cells 'home' to the bone marrow via a SDF-1α gradient, migrate across the local vasculature, and ultimately reside near the endosteum (4). Mesenchymal stem cells (MSC) and bone marrow–resident stem cells are immune suppressors and might protect the cancer cells from the immune system in the bone marrow, thereby facilitating their survival in the bone marrow (33, 34). MSCs surround the abluminal region of bone marrow vasculature and therefore could regulate transendothelial migration of BCCs and concomitantly suppress an immune response. Homing, transendothelial migration, and tumor cell quiescence are tremendously complex processes; how the bone marrow microenvironment induces these changes is unclear but proposed mechanisms include gap junction formation, decreased oxygen tension, quiescence-promoting cytokine profiles, microRNAs, etc. We have previously reported changes in cytokine production once the BCCs form contact with stromal cells (22). Although we cannot explain why these cytokines are regulated to balance the negative and positive hematopoietic effects, multiple mechanisms discussed above could be operative. Once the homeostasis of dormancy has been established, any disruption may predispose a patient to reemerging cancer. Potential disruptions could include local infections or reaction to infection, bone marrow aging, trauma, or medications. To approach truly 'curative' therapeutic outcomes, continued work is required to tease out mechanisms regulating tumor quiescence and resurgence.

In summary, this study adds to the potential roles of neurokinin receptors in breast cancer development and, perhaps, other cancers (35). The cartoon, shown in Fig. 6, adds previous studies on neurokinin receptors to the current findings to summarize the interactions among neurokinin receptors and *Tac1* peptides in BCCs (20–22). *Tac1* peptides, which are constitutively produced in BCCs, interact with neurokinin receptors to induce the production of several cytokines. This led to autocrine stimulation by cytokines and/or Tac1 peptides and activation of NF-κB. The latter transcription factor, through unresolved mechanism, is relevant to

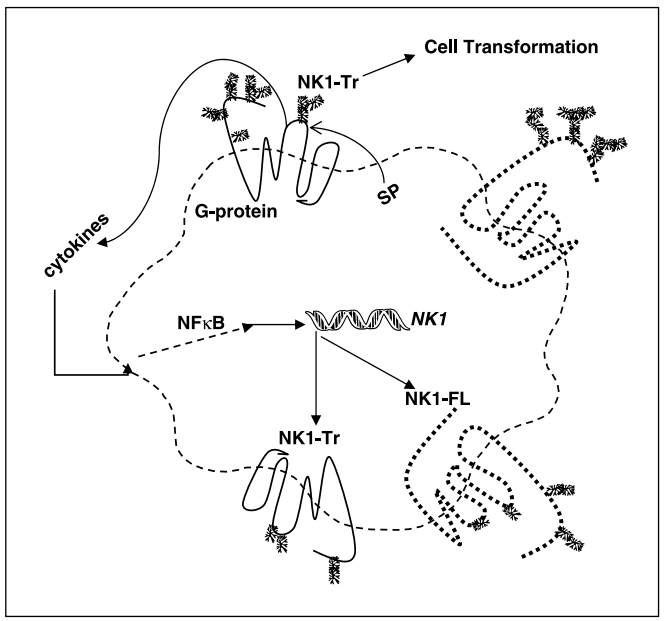

Figure 6. Cartoon summarizing a central role for NF-κB in regulating NK1 expression in BCCs. The ligands from *Tac1* [substance P (*SP*)], highly expressed in BCCs, interact with NK1 subtypes to induce cytokines. Cytokine stimulation then leads to activation of NF-κB, which mediates the induction of *NK1* resulting in membrane expression of NK1-FL and NK1-Tr.

the expression of NK1-Tr. This study forms the impetus for future *in vivo* and *in vitro* studies. Further studies are ongoing to understand the mechanisms of the transforming abilities of NK1-Tr. Because BCCs show preference for the bone marrow, tracing studies are needed to determine if NK1-Tr and/or NK1-FL are relevant for BCC metastasis to the bone marrow. Studies are needed to address their roles within the bone marrow cavity and while the BCCs enter the bone marrow through the vasculature/mesenchymal barrier. Ultimately, this study will form the bridge toward understanding the reasons by which BCCs remain undetectable during the early stages of metastasis.

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Nuclear Factor-κB Is Central to the Expression of Truncated Neurokinin-1 Receptor in Breast Cancer: Implication for Breast Cancer Cell Quiescence within Bone Marrow Stroma

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