

Nuclear factor- κ B activation: a molecular therapeutic target for estrogen receptor–negative and epidermal growth factor receptor family receptor–positive human breast cancer

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Abstract

Nuclear factor- κ B (NF- κ B), a transcription factor with pleiotropic effects, is a downstream mediator of growth signaling in estrogen receptor (ER)-negative and erbB family particularly erbB2 (HER-2/*neu*) receptor–positive cancer. We previously reported activation of NF- κ B in ER-negative breast cancer cells and breast tumor specimens, but the consequence of inhibiting NF- κ B activation in this subclass of breast cancer has not been shown. In this study, we investigated the role of NF- κ B activation by studying the tumorigenic potential of cells expressing genetically manipulated, inducible, dominant-negative inhibitory κ B kinase (IKK) β in xenograft tumor model. Conditional inhibition of NF- κ B activation by the inducible expression of dominant-negative IKK β simultaneously blocked cell proliferation, reinstated apoptosis, and dramatically blocked xenograft tumor formation. Secondly, the humanized anti-erbB2 antibody trastuzumab (Herceptin) and the specific IKK inhibitor NF- κ B essential modifier–binding domain peptide both blocked NF- κ B activation and cell proliferation and reinstated apoptosis in two ER-negative and erbB2-positive human breast cancer cell lines that are used as representative model systems.

Combinations of these two target-specific inhibitors synergistically blocked cell proliferation at concentrations that were singly ineffective. Inhibition of NF- κ B activation with two other low molecular weight compounds, PS1145 and PS341, which inhibited IKK activity and proteasome-mediated phosphorylated inhibitory κ B protein degradation, respectively, blocked erbB2-mediated cell growth and reversed antiapoptotic machinery. These results implicate NF- κ B activation in the tumorigenesis and progression of ER-negative breast cancer. It is postulated that this transcription factor and its activation cascade offer therapeutic targets for erbB2-positive and ER-negative breast cancer. [Mol Cancer Ther 2007;6(7):1973–82]

Introduction

Human breast cancer is not a single disease but a composite of disorders of mammary epithelial cells, with distinct pathologic characteristics and diverging clinical manifestations (1, 2). Human breast cancers are grouped according to the expression levels of estrogen receptor (ER), progesterone receptor, and the epidermal growth factor (EGF) family, principally the erbB2 (HER-2/*neu*) receptor protein (3). Enhanced cell proliferation and reduced regulated cell death (apoptosis) characterize the malignant phenotype and summarize the several functions of the transcription factor nuclear factor- κ B (NF- κ B).

Elevated levels of NF- κ B are frequently detected in many diseases, including breast cancer (4–6). This transcription factor was discovered in immune cells and believed to be involved primarily in the transmission of inflammatory signals by modulation of the expression of immune response genes (7). Later, NF- κ B was discovered to be present ubiquitously in most cell types in an inactive state, in complex with the inhibitory κ B protein (I κ B) in the cytoplasm (8, 9). NF- κ B is a heterodimeric complex of *rel* family proteins, and its activation transduces cell signals from diverging molecules, such as cytokines, mitogens, growth factors, and bacterial and viral gene products (9). The heterodimer of p65 (*rel* A) and p50 is the predominant active NF- κ B complex in epithelial cells. Activated NF- κ B induces multiple cellular functions, including enhanced cell proliferation and reduced apoptosis. Activation of NF- κ B proceeds by phosphorylation of I κ B by inhibitory κ B kinase (IKK), releasing the heterodimeric active complex, which is translocated to the nucleus and binds to specific DNA sequences and transactivates responsive genes (7, 9–12). The phosphorylated I κ B is rapidly modified by ubiquitylation and degraded in proteasomes.

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Activation of NF- κ B in human breast cancer is confined predominately to the ER-negative subtype of cancers, particularly those that express members of the EGF family of receptors, including the EGF receptor (erbB1) and erbB2 (HER-2/*neu*; refs. 6, 12). This trend was confirmed in tissue samples from patients with breast cancer (6). NF- κ B activation is dependent on growth factors interacting with their specific receptors in these cell types. The signal is induced within minutes following treatment with EGF in EGF receptor overexpressing cells and with heregulin β 1 (HRG), the ligand for erbB3 and erbB4, in erbB2-expressing breast cancer cells (6, 12–14).

Current therapy for erbB2-positive breast cancer is directed at the receptor-like molecule (13, 14). Trastuzumab (Herceptin), a humanized monoclonal antibody directed against erbB2, has clinical activity in both early and advanced breast cancers that contain an amplification of the *erbB2* gene. Recent studies have evaluated chemotherapy with and without trastuzumab after surgery for women with erbB2-positive breast cancer (13–15). In metastatic breast cancer, the addition of trastuzumab to chemotherapy lengthens the time to disease progression, improves the response rates to therapy, and prolongs survival (16). However, both *de novo* and acquired resistance to trastuzumab is prevalent, and overall survival gains have yet to be realized with trastuzumab monotherapy. Therefore, more effective combinations containing trastuzumab are sought for erbB2-expressing breast cancer. We propose that inhibitors of NF- κ B-activating kinase (IKK) would be potential candidates for combination therapy for ER-negative and erbB2-positive breast cancer patients.

The association of NF- κ B activation with subclasses of human breast cancer provides circumstantial evidence for its role in carcinogenesis. In this study, we used several approaches to block NF- κ B activation in ER-negative breast cancer cells and investigated the consequences on cell proliferation, apoptosis, and xenograft tumor formation. Several cell lines, including ER negative and erbB2 positive (SKBr3 and MDA-MB453), ER negative and erbB1 positive (MDA-MB231), and ER positive and erbB1/erbB2 negative (MCF-7), were used as representative cultured cell models for subclasses of human breast cancer. We used a conditional dominant-negative gene construct and small-molecule inhibitors to disable IKK, the key kinase of NF- κ B activation. We also used the proteasome inhibitor bortezomib to inhibit NF- κ B activation. These inhibitors were effective in erbB2-positive and ER-negative breast cancer cells, which harbor activated NF- κ B, but had little or no effect in ER-positive cells. As a practical application, we also found that treatment of cells with trastuzumab combined with drugs that inhibit NF- κ B blocked cell proliferation at concentrations that are incapable of exerting significant inhibitory effects when used singly. The genetically manipulated inducible dominant-negative IKK β (dnIKK β) regulated the level of activated NF- κ B in the host cells and affected the downstream events. These constructs were introduced into erbB1-overexpressing ER-

negative breast cancer cells (MDA-MB231), and the behavior of xenografts initiated by the altered cells was observed. Our results underscore the importance of competent NF- κ B activation for the growth of certain breast cancer subtypes and qualify NF- κ B activation as a novel therapeutic target for the subgroup of ER-negative breast cancer patient.

Materials and Methods

Materials

Human breast cancer cell lines were obtained from the American Type Culture Collection and grown in rich medium (RPMI 1640 with 10% fetal bovine serum; ref. 6). The minimal medium is RPMI 1640 without phenol red supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (Hyclone). HRG and anti-actin antibody are from Sigma Chemical; trastuzumab (marketed as Herceptin, Genentech) is a gift from Dr. Lindsay Harris (Yale University, New Haven, CT). Anti-I κ B α , anti-phosphorylated I κ B α , and anti-p65 antibodies are from Cell Signaling; anti-FOXO3A, anti-phosphorylated FOXO3A, and anti-IKK γ antibodies are from Santa Cruz Biotechnology. The NF- κ B essential modifier-binding domain peptide (NBD) is synthesized at Dana-Farber Cancer Institute (17, 18). PS1145 and PS341 are provided by Millennium Pharmaceuticals. The NF- κ B-Luciferase *cis*-Reporting System is from Stratagene.

Activation of NF- κ B

Active NF- κ B is defined by DNA-binding activity in nuclear extracts from control and treated cells measured by electrophoretic mobility shift assay (EMSA) and quantitated by scanning densitometry (6). The functional state of NF- κ B activation was also measured by a NF- κ B *cis*-reporting system expressing luciferase with a β -actin promoter-driven *Renilla* luciferase vector (Dual-Luciferase Assay System, Promega; ref. 19).

IKK Activity

IKK activity was measured by immunoprecipitation of the IKK complex with IKK γ antibody in whole-cell extracts of control and treated cells followed by incubation with purified recombinant I κ B α -glutathione *S*-transferase fusion peptide (1–54 COOH-terminal amino acids with the two target serine residues) in the presence of [γ - 32 P]ATP. Fractionation and detection of 32 P-I κ B α products by autoradiography and nonradioactive substrate by staining with Coomassie blue as described previously (17, 18).

Synergistic Effects of Drug Combinations

Additive, synergistic, or antagonistic effects of drug combinations were determined by the method of Chou and Talalay (20, 21) and analyzed by calculating the combination index (CI). CI of <0.1, <0.3, and <0.7 is considered as very strongly synergistic, strongly synergistic, and synergistic interaction of the combination, respectively. A CI of 0.9 to 1.0 is considered as additive and CI of >1.0 is considered antagonistic. We used the CalcuSyn Program for Windows (Biosoft) to compute CI values.

Xenograft Nude Mice Tumor Model

Human breast cancer cells (2×10^6 to 3×10^6) were suspended in 0.5 mL sterile PBS and implanted under the dorsal skin of 6-week-old female *nu/nu* mice (NCR NUM Homozygote, Taconic). Tumor growth was monitored by periodic measurement of volume using the modified formula $SS^2 \times LS \times 0.41$ (SS is the short side and LS is the long side; ref. 22). Doxycycline (DOX; Sigma-Aldrich) was given as a drinking solution in light-protected bottles (5% sucrose solution with 0.5 mg/mL DOX). The control animals received only the 5% sucrose solution.

Results and Discussion

Differential Activation of NF- κ B in Subclasses of Breast Cancer Cells

An elevated level of activated NF- κ B is detected in specific subclasses of human breast cancer cells and tumor tissue specimens, predominantly in erbB2-overexpressing ER-negative breast cancer (5, 6, 12). We studied the NF- κ B activation pattern in ER-negative and erbB2-positive

SKBr3 and MDA-MB453 cell lines as representative working systems of this subclass of human breast cancer and compared the results to the ER-positive and erbB2-negative human breast cancer cell line MCF-7. The level of activated NF- κ B in SKBr3 could be experimentally regulated by growing the cells in depleted (minimal) medium followed by supplementation with the specific mitogenic growth factor HRG (+HRG). This treatment elevated the level of IKK activity as early as 15 min with simultaneous elevation of the NF- κ B DNA-binding activity and NF- κ B-driven reporter gene expression (Fig. 1A). These results suggest that IKK-mediated NF- κ B activation participates in HRG-initiated signaling in erbB2-positive cells. Similar results were observed in a second erbB2-positive and ER-negative breast cancer cell line MDA-MB453 (see below).

In contrast, treatment of MCF-7 cells with 17 β -estradiol (E2) for as short as 15 min substantially stimulated IKK enzyme activity but failed to modulate the basal NF- κ B activation and downstream reporter gene expression even

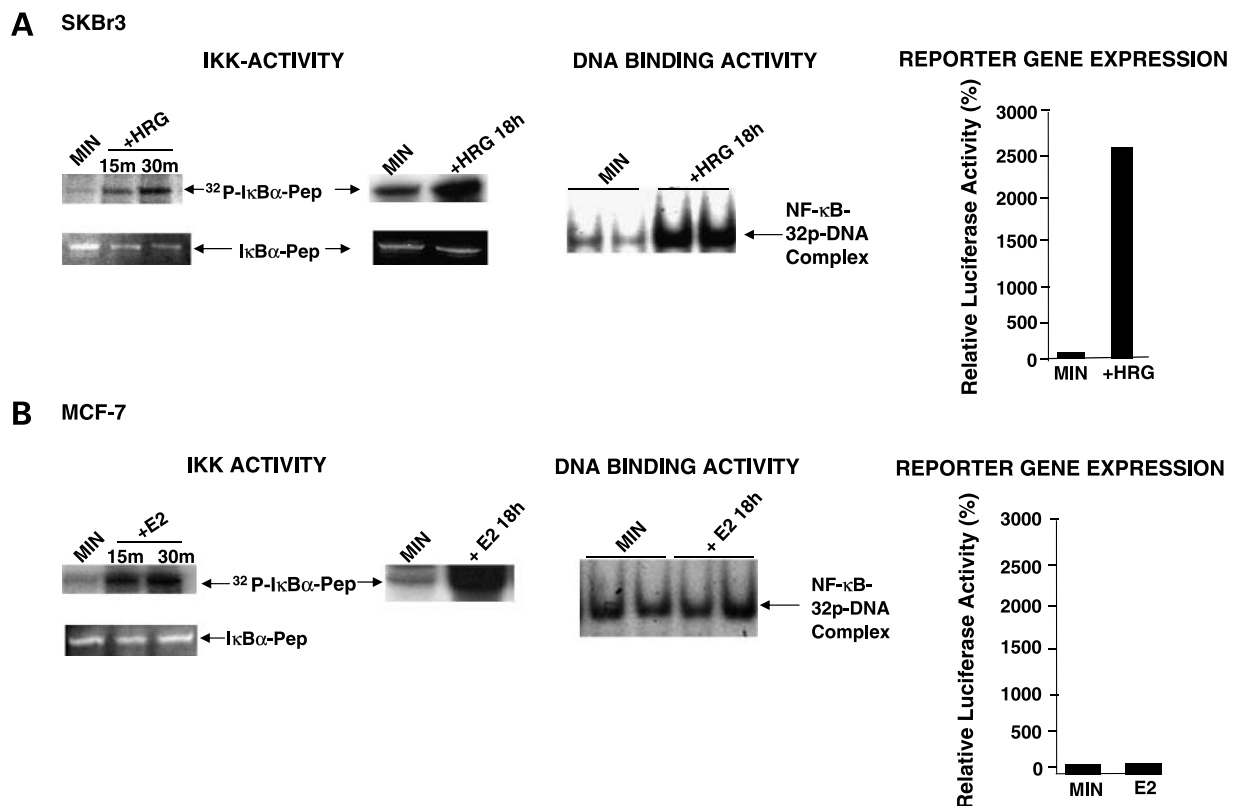


Figure 1. Differential regulation of NF- κ B activation in human breast cancer cells. **A**, SKBr3 cells. IKK activity: IKK in whole-cell extracts (200 μ g) of SKBr3 cells grown in minimal medium (MIN) and in the presence of HRG (+HRG; 2 nmol/L) for the indicated period (15 min, 30 min, and 18 h) was immunoprecipitated with anti-IKK γ antibody. *Top*, level of autoradiographic signals of ³²P-phosphorylated IκB α -peptide (³²P-IκB α -Pep) product in the incubation mixture; *bottom*, amount of the total added substrate IκB α peptide (stained with Coomassie blue) in the same reaction mixture. NF- κ B DNA-binding activity: nuclear extracts (5 μ g) from SKBr3 cells grown in minimal medium and in the presence of HRG (2 nmol/L, for 18 h) were prepared and NF- κ B-binding activity was determined by EMSA. NF- κ B *cis*-reporter gene expression: expression of luciferase gene linked with NF- κ B response elements was determined in extracts of transfected cell (19). The results are presented as a percentage of actin promoter-driven *Renilla* luciferase activity. **B**, MCF-7 cells. MCF-7 cells were grown in minimal medium and minimal medium + E2 (+E2; 100 nmol/L) for the indicated period (15 min, 30 min, and 18 h). IKK activity in 200 μ g of cell extracts, NF- κ B DNA-binding activity in nuclear extracts (5 μ g), and NF- κ B *cis*-reporter gene expression were determined as in **A**.

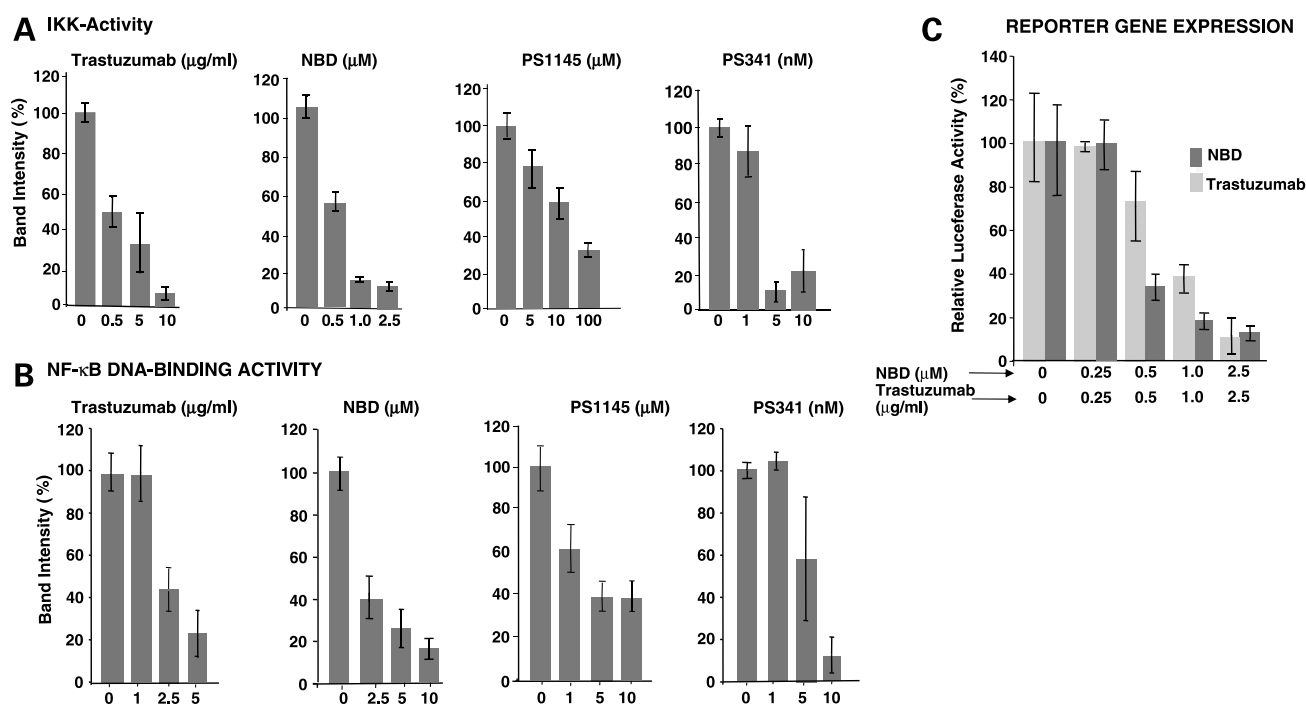


Figure 2. Influence of erbB2 and NF- κ B inhibitors. **A**, IKK activity. SKBr3 cells were grown in minimal medium and minimal medium + HRG (2 nmol/L) for 18 h in the presence or absence (0) of the specified drug at the indicated concentrations. IKK in the whole-cell extracts was immunoprecipitated with anti-IKK γ antibody and IKK activity was quantitated by densitometric scanning of the autoradiographic signals. The influence of the inhibitors on IKK activity is expressed as a percentage of that in the presence of HRG alone. **B**, NF- κ B DNA-binding activity. Nuclear extracts were prepared from SKBr3 cells grown in minimal medium + HRG (2 nmol/L) for 18 h in the presence or absence (0) of indicated concentrations of inhibitors. The NF- κ B DNA-binding activity in control and treated nuclear extracts was measured by EMSA and quantitated as described in **A**. Results are expressed as percentage of the signal detected in the same amount of nuclear extracts (5 μ g protein) from cells grown in HRG alone. **C**, NF- κ B-driven reporter gene expression. SKBr3 cells were grown in HRG (2 nmol/L) containing medium for 18 h in the presence of trastuzumab and NBD at the indicated concentrations. The NF- κ B-driven reporter gene activity was measured and expressed as a percentage of the maximal expression in cells treated with HRG alone.

after 18 h of exposure to E2 (Fig. 1B). The observed robust activity of IKK with short-term E2 treatment is consistent with a cell signaling event rather than just a consequence of E2-mediated cell proliferation. The differential activation pattern between ER-negative and ER-positive breast cancer cells and absence of regulatable activated NF- κ B in another class (Fig. 1B) suggested a potential role of this transcription factor in the class in which it is activated.

There is two-way communication between estrogen signaling and the NF- κ B pathway. In one direction, estrogen signaling is potentiated by IKK α through its phosphorylation of the estrogen receptor, which is recruited to estrogen-responsive promoters after E2 stimulation (23). In the other direction, E2 is a potent inhibitor of NF- κ B signaling and is dependent on ER (24). We did not exhaustively examine the effects of E2 on canonical NF- κ B signaling in MCF-7 cells; however, IKK α activity stimulated by E2 did not result in an increased level of cytoplasmic phosphorylated I κ B (data not shown). In contrast, erbB2-positive and ER-negative cells rapidly induced a NF- κ B signal in the presence of HRG. Inability to up-regulate NF- κ B by E2 in ER-positive cells even in the presence of activated IKK is intriguing. This differential role of IKK α in phosphorylating nuclear ER and cytoplasmic I κ B may play

a crucial role in the cross-talk between ER-positive and ER-negative breast cancer cells.

Inhibition of NF- κ B Activation

Because erbB signals are transmitted through IKK-catalyzed NF- κ B activation (6, 12), we investigated the ability to interrupt the pathway by blocking the activating kinase with specific inhibitors. The inhibitors of the NF- κ B pathway were compared with trastuzumab, a monoclonal antibody to erbB2, which interrupts upstream transmission of signals from the cell surface receptor to cytoplasmic IKK (6). The NBD contains 16 amino acids spanning the COOH-terminal region of IKK β to which NF- κ B essential modifier (IKK γ) binds to fully activate the IKK complex. NBD contains additional 17 amino acids from *Drosophila antennapedia* to render the peptide permeable (17, 18). PS1145 is a chemical inhibitor of IKK with IC₅₀ of 88 nmol/L with no appreciable inhibitory activity against 14 other kinases (25). PS341 (bortezomib) is a proteasome inhibitor that, among other actions, inhibits the degradation of I κ B and prevents activation of NF- κ B (25, 26).

The IKK activity was examined in HRG-stimulated and NBD-, trastuzumab-, PS1145-, and PS341-treated SKBr3 cells. NBD peptide, the IKK-specific inhibitor, and trastuzumab, the anti-erbB2 antibody, and the two low molecular

weight compounds showed similar patterns of concentration-dependent inhibition (Fig. 2A). The inhibition of IKK activity by these compounds strictly correlated with simultaneous reduction in HRG-induced NF- κ B DNA-binding activity (Fig. 2B) and NF- κ B response element-driven reporter gene expression (Fig. 2C). These effects detected at 18 h also were apparent after 2 h of treatment with the IKK inhibitors (6). In contrast, these inhibitors did not show any influence on the basal level of NF- κ B DNA-binding activity in MCF-7 cells (data not shown). Comparable results that are observed in SKBr3 cells were duplicated in a second erbB2-positive and ER-negative human breast cancer cell line MDA-MB453 (see below). Based on the similar time frame and dose response of trastuzumab and small molecular IKK inhibitors, we conclude that signaling from EGF receptor (EGFR) family receptors is transmitted via IKK and stimulated NF- κ B activation as determined by DNA-binding activity. We and others showed that drugs blocking NF- κ B activation in ER-negative breast cancer cells do not affect the constitutively activated NF- κ B in ER-positive cells, suggesting an alternative mechanism of NF- κ B activation in the latter cell types (6, 27, 28).

Synergistic Inhibition of Proliferation by erbB2 and NF- κ B Inhibitors

The consequence of interrupting erbB2 and NF- κ B signaling on cell proliferation was studied by assessing

combinations of drugs specifically targeting the erbB2 receptor and NF- κ B activation cascades. Reciprocal experiments were conducted with a constant concentration of one agent in combination with variable concentrations of the second. Cell proliferation assays were conducted with variable drug concentrations while maintaining a predetermined constant concentration ratio. From these results, synergistic or additive effects on cell proliferation were determined (20, 21).

The influence of trastuzumab and NBD was examined in ER-negative and erbB2-amplified SKBr3 (Table 1) and MDA-MB453 cells (Table 2). Although trastuzumab was effective in inhibiting the proliferation of SKBr3 cells, it had no effect against MDA-MB453 cells at doses up to 20 μ g/mL. In SKBr3 cells, combinations of NBD and trastuzumab at concentrations as low as 0.1 μ mol/L and 0.2 μ g/mL, respectively, showed a strong synergistic effect (CI value, 0.024) on cell proliferation (Table 1). Similar synergistic effects of combinations of PS1145 and trastuzumab (CI value, 0.112–0.115) at the ratio of 1:2 and PS341 and trastuzumab (CI value, 0.329–0.349) at the ratio of 1:1 (Table 1) were observed.

Because trastuzumab by itself was incapable of inhibiting proliferation in MDA-MB453 cells, its influence in combination with other inhibitors could not be measured. However, HRG elevated NF- κ B DNA-binding activity in MDA-MB453 cells (Fig. 3A). The IKK inhibitors NBD and

Table 1. Synergistic inhibition of SKBr3 cell proliferation by combinations of drugs

Drug combinations		CI*		Synergy score [†]
NBD (μ mol/L) [‡]	Trastuzumab (μ g/mL)	Exclusive	Nonexclusive	
0.1	0.2	0.024	0.024	Very strong
0.25	0.5	0.035	0.035	Very strong
0.5	1.0	0.036	0.036	Very strong
1.0	2.0	0.117	0.117	Strong
2.5	5.0	0.193	0.193	Strong
5.0	10.0	0.388	0.388	Synergistic
PS1145 (μ mol/L)	Trastuzumab (μ g/mL)			
0.5	1.0	0.112	0.115	Strong
1.0	2.0	0.016	0.016	Very strong
2.5	5.0	0.121	0.124	Strong
5.0	10.0	0.242	0.255	Strong
PS341 (nmol/L)	Trastuzumab (μ g/mL)			
0.5	1.0	0.329	0.349	Synergistic
2.0	2.0	0.189	0.198	Strong
5.0	5.0	0.154	0.159	Strong
10.0	10.0	0.714	0.841	Moderate
20.0	20.0	0.118	0.120	Synergistic

*CI calculated by the method of Chou and Talalay (20, 21) using CalcuSyn software. Mutually exclusive CI values are for drugs that have the same or similar modes of action. Nonexclusive drugs are ones with independent modes of action.

[†]Synergy score is defined as follows: CI < 0.1, very strongly synergistic; 0.1 < CI < 0.3, strongly synergistic; 0.3 < CI < 0.7, synergistic; 0.7 < CI < 0.85, moderately synergistic.

[‡]Drugs are NBD, trastuzumab, PS1145 (IKK inhibitor), and PS341 (marketed as Velcade).

PS1145, the proteasome inhibitor PS341, and trastuzumab all blocked NF- κ B DNA-binding activity in MDA-MB453 cells (Fig. 3B). The IKK inhibitors and PS341 alone blocked cell proliferation. Combinations of NBD and PS341 at 1:1 ratio showed strong synergism with CI value of 0.280 to 0.298 in this ER-negative and erbB2-positive cell line (Table 2). Thus, it is possible that breast cancers that fail to respond to trastuzumab by itself (exemplified by MDA-MB453) may be treated with combinations of IKK inhibitors, such as NBD, and proteasome inhibitors, such as PS341.

Both NBD and PS1145 inhibited proliferation of erbB2-positive and ER-negative breast cancer cells alone or acted synergistically when combined with trastuzumab in SKBr3 cells.

These findings have at least two implications. First, NF- κ B activation seems to be a component of growth signaling pathways in erbB2-positive and ER-negative breast cancer cells, perhaps downstream of an information signal traveling through phosphatidylinositol 3-kinase and AKT phosphorylation (24, 29). Second, combination therapy based on trastuzumab, and perhaps other small-molecule inhibitors of erbB family receptors, may benefit from the addition of NF- κ B-directed drugs. Detection of activated

NF- κ B in erbB2-positive and ER-negative breast cancers, the absence of activation in ER-positive cancers, and the inhibition of erbB2-mediated cell proliferation make a strong case for the critical role of NF- κ B in certain human breast cancers.

Dual Role of IKK-Mediated Regulation of Cell Proliferation and Apoptosis

Activation of antiapoptotic NF- κ B and inactivation of the proapoptotic FOXO3A, one of the members of the forkhead family proteins, are mediated by IKK (30, 31). Phosphorylation of FOXO factors leads to their release from DNA, sequestration in the cytoplasm, and ubiquitin-mediated proteasome degradation. The net result of these events is the release from growth arrest and suppression of apoptosis.

We have shown previously that IKK-regulated SKBr3 cell proliferation and apoptosis are mediated via activation of NF- κ B. In this study, we provide additional data to show that IKK-mediated inactivation of FOXO3A, the functionally opposite transcription factor, also resulted in similar effects on cell proliferation and apoptosis. HRG-induced activation of IKK caused phosphorylation of I κ B that released active NF- κ B and simultaneously phosphorylated and inactivated FOXO3A. In rich medium, both I κ B α and FOXO3A are phosphorylated as evidenced by reduced level of I κ B and increased level of phosphorylated I κ B (Fig. 4A, rows 1 and 2) and reduced FOXO3A and increased phosphorylated FOXO3A (Fig. 4A, rows 3 and 4). Switching to minimal medium reduced both the phosphorylated species of I κ B α and FOXO3A. Stimulation of NF- κ B activation by treatment with HRG once again restored phosphorylated I κ B α and FOXO3A. These modifications were detectable at 2 h of HRG treatment (6). Both NBD and trastuzumab simultaneously blocked HRG-stimulated and IKK-mediated phosphorylation of I κ B α and FOXO3A (Fig. 4A).

Coincident with the molecular events shown in Fig. 4A, cell proliferation and apoptosis responded reciprocally both by altering the growth conditions and by specific inhibitors (Fig. 4B and C). Cell proliferation as measured by percentage viable cells was elevated in rich medium, reduced in minimal medium, and stimulated in HRG-supplemented minimal medium. Either blocking erbB2 signaling with trastuzumab or blocking IKK signaling with NBD inhibited the HRG-mediated stimulation of cell proliferation (Fig. 4B). The apoptotic fraction, in contrast, was low in rich medium, elevated by shifting to minimal medium, and returned to its baseline by treatment with HRG (Fig. 4C). Treatment with either trastuzumab or NBD reduced proliferation and increased apoptosis. Consistent with these results, treatment with either PS1145 or PS341 increased the apoptotic fraction of cells by a similar amount. Treatment of cells with inhibitors blocked cell cycle progression, reduced the S-phase fraction, and caused accumulation of cells in G₁ (Fig. 4D). The net results of the activation and inactivation of NF- κ B and FOXO3A, respectively, are the enhanced cell proliferation and reduced apoptosis, two characteristic phenotypes of cancer

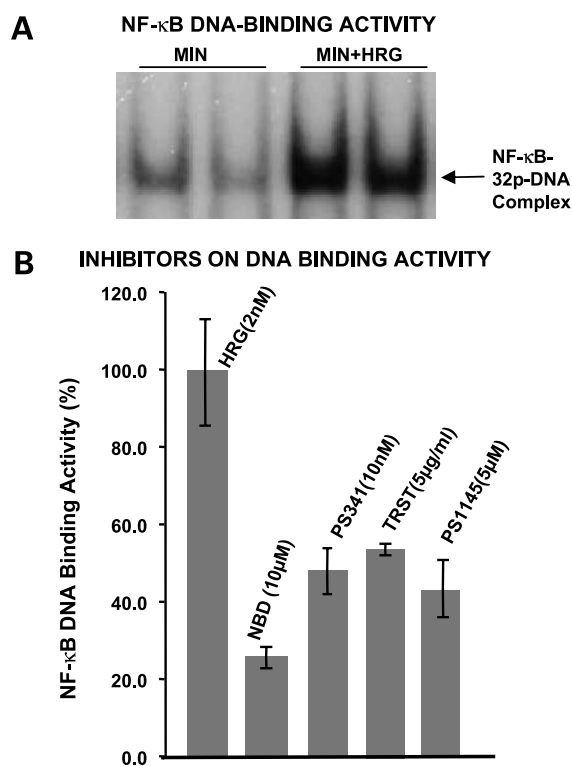


Figure 3. Influence of inhibitors of NF- κ B activation in MDA-MB453 cells. **A**, DNA-binding activity. NF- κ B DNA-binding activity in the nuclear extracts of MDA-MB453 cells grown in minimal medium and minimal medium + HRG (2 nmol/L) was studied by EMSA. **B**, influence of inhibitors. Influence of low molecular weight inhibitors on NF- κ B activation was determined by EMSA at the indicated concentrations of the listed compounds. Results of average (with error bars) of triplicate samples for each drug treatment in three experiments. TRST, trastuzumab.

Table 2. Inhibition of MDA-MB453 cell proliferation by combinations of NBD and PS1145

Drug concentrations		CI*		Synergy level
NBD ($\mu\text{mol/L}$)	PS1145 ($\mu\text{mol/L}$)	Exclusive	Nonexclusive	
0.5	0.5	0.280	0.298	Strong synergism
1.0	1.0	0.223	0.236	Strong synergism
2.5	2.5	0.562	0.641	Synergism
5.0	5.0	0.665	0.768	Moderate synergism

NOTE: Determination of CI by Chou-Talalay method: cell proliferation was monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (41) in the presence of different concentrations of NBD and PS114 either singly or in combinations. The predetermined ratio of drug concentrations was 1:1. Synergistic effect of drug concentrations on cell proliferation is estimated from the CI calculated by the Chou and Talalay method (20, 21).

*Exclusive CI values implicate drugs with similar modes of action and nonexclusive CI values implicate drugs with independent modes of action (CI < 0.1, very strongly synergistic; CI = 0.1–0.3, strongly synergistic; CI = 0.3–0.7, moderately synergistic).

cells. The converse, reduction of phosphorylated FOXO3A and the accumulation of total FOXO3A protein after specific inhibition of IKK by NBD, is consistent and predicted IKK stands at a central fork in cell fate, proliferation, or cell death, and a picture emerges of a critical signaling cascade in cancer cells (Fig. 4E). Growth factors send signals through IKK, which has dual but contrasting effects on cell proliferation and apoptosis by signaling the degradation of I κ B α and FOXO3A. Consequently, NF- κ B is released from I κ B block and activated and translocated to the nucleus that transactivates pro-proliferative and antiapoptotic target genes. Simultaneously, phosphorylation degrades the proapoptotic FOXO3A. Drugs acting on IKK or on upstream intermediates have dual and opposing effects by freezing NF- κ B in inactive state and simultaneously maintaining FOXO3A in active state, which is to reduce cell proliferation and reinstate apoptosis. Figure 4E does not depict the contributions of phosphatidylinositol 3-kinase, which may be the most active upstream effector of NF- κ B activation and FOXO3A inactivation (31, 32). However, in addition to phosphatidylinositol 3-kinase, IKK controls important cell fates and is a kinase worthy of pharmaceutical attack in breast cancers with an active NF- κ B pathway.

Outcome of Blocking IKK-Mediated NF- κ B Activation

The role of NF- κ B activation in human breast cancer was further examined by blocking its activation in ER-negative MDA-MB231 cells via stable integration of an inducible dnIKK β allele (32). We chose MDA-MB231 because these cells grow rapidly as xenografts relative to some other poorly tumorigenic breast cancer cell lines, such as SKBr3 and MCF-7. FLAG-tagged dnIKK β -coding DNA construct was inserted in the inducible tet-OFF/ON expression system. The dnIKK β is expressed in the absence of DOX (DOX⁻), allowing experimental manipulation of the dominant-negative allele (Supplementary Fig. S1).⁵ The level of active NF- κ B in MDA-MB231 cells can be down-regulated by growing cells in minimal medium and enhanced by

adding EGF to nonpermissive growth medium (33). Treatment with HRG did not influence the basal activity of NF- κ B in MDA-MB231 cells that is consistent with its low levels of erbB2 (Fig. 5A).

Clones 26 and 27 of MDA-MB231 containing dnIKK β are two clones in which the level of activated NF- κ B could be modulated by the presence (DOX⁺) or absence (DOX⁻) of DOX in the cell growth medium (Fig. 5B). Growing either clone in rich medium and in the presence of DOX (DOX⁺) maintains the level of active NF- κ B as determined by EMSA, presumably mediated via wild-type cellular IKK. Removal of DOX (DOX⁻) permits expression of dnIKK β and, under its influence, down-regulated NF- κ B activation in both the clones (Fig. 5B). Treatment of the cells with the IKK-specific inhibitor NBD did not further reduced the basal level of activated NF- κ B in DOX⁻ medium, suggesting that IKK is not involved in the regulation of the basal level of activated NF- κ B (data not shown). These cells growing in DOX⁻ contained a higher fraction of apoptotic cells that was reduced by adding DOX to the growth medium (Fig. 5C). As expected, the clones grow exponentially in DOX⁺ medium and are reduced in DOX⁻ medium (Fig. 5D, *left*). Cell proliferation and apoptosis were unaffected by DOX withdrawal in stable transfectants of MDA-MB231 cells with vector plasmid alone (Fig. 5D, *right*).

The expression of dnIKK β dramatically affected the tumorigenic potential of MDA-MB231 cells in nude mice (Fig. 5E). Parental MDA-MB231 and vector-containing cells formed tumors at the same rate in the presence or absence of DOX (Supplementary Table S1).⁵ Xenograft tumors with clone 26 were detected in the presence of DOX (DOX⁺) in tet-OFF mode (Supplementary Table S1; Fig. 5E).⁵ However, no tumors were detected even after 30 weeks when clone 26 cells were implanted in the absence of DOX (DOX⁻) that allowed expression of dnIKK β (Supplementary Table S1; Fig. 5E).⁵ Thus, these results show that incapacitating NF- κ B signaling prevents xenograft tumor growth of cells that otherwise readily form tumors in recipient animals, suggesting that active NF- κ B potentiates and is necessary for tumorigenesis in this subclass of human breast cancer cells.

It is not surprising that NF- κ B fulfills an important role in human breast cancer. In many cell types, NF- κ B integrates

⁵ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

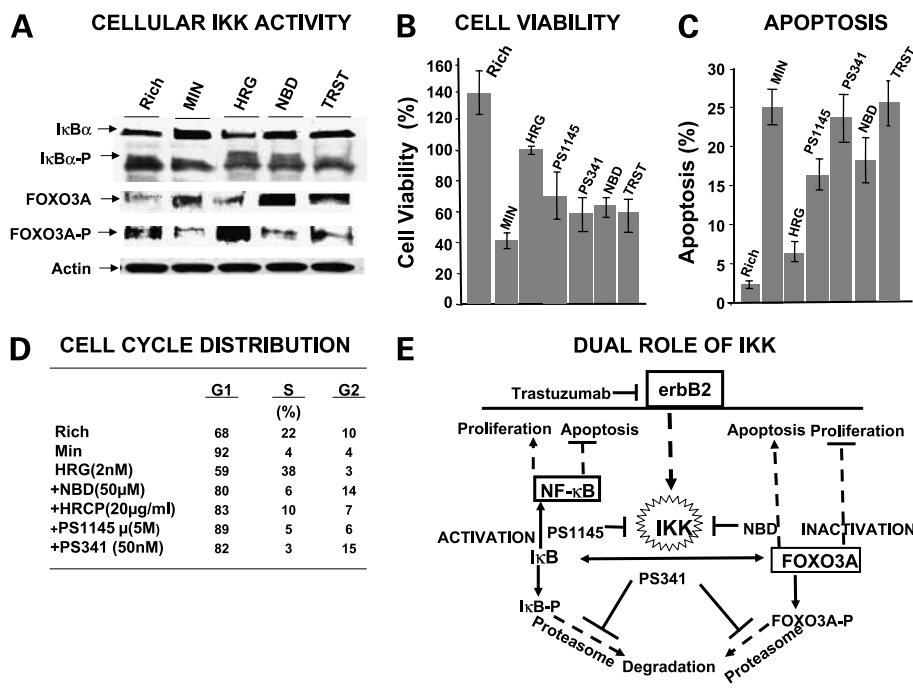


Figure 4. IKK-mediated phosphorylation of I κ B α and FOXO3A. **A**, cellular IKK activity. IKK activity in treated and control SKBr3 cells was monitored by measuring the levels of total cellular I κ B α , phosphorylated I κ B α (I κ B α -P), total FOXO3A, and phosphorylated FOXO3A (FOXO3A-P) by Western blot analysis of whole-cell extracts (50 μ g). SKBr3 cells were grown in rich medium (Rich), minimal medium, and minimal medium + HRG (2 nmol/L) or + NBD (10 μ mol/L) or + trastuzumab (10 μ g/mL) for 18 h. Actin in the same amount of cell extract served as loading control. **B**, cell viability. Viability of SKBr3 cells in rich medium, minimal medium, and minimal medium supplemented with HRG (2 nmol/L) in HRG + PS1145 (10 μ mol/L) or + PS341 (5 nmol/L) or + trastuzumab (10 μ g/mL) or + NBD (10 μ mol/L) was measured after 5 d of treatment. Cell viability as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay with different drugs relative to HRG treatment (100%). **C**, apoptosis. The percentage of apoptotic cells was determined by Annexin binding (6). SKBr3 cells were grown in rich medium, minimal medium, and minimal medium in the presence of the indicated drugs at the same concentrations and treatment conditions used in **B**. **D**, cell cycle distribution. SKBr3 cells were grown in rich medium or minimal medium in the presence of activator and inhibitors at the indicated concentrations for 18 h. Cells in different phases of cell cycle were measured by propidium iodide staining followed by fluorescence-activated cell sorting analysis according to the procedure provided by the core facilities of Dana-Farber Cancer Institute and expressed as percentage of the total (6). **E**, schematic presentation of dual role of IKK. IKK-mediated phosphorylation and activation of antiapoptotic NF- κ B and inactivation of apoptotic FOXO3A. Phosphorylation of I κ B in the NF- κ B-I κ B complex releases phosphorylated I κ B and targets its degradation by the ubiquitinylation-proteasome pathway. Released active NF- κ B complex then enters the nucleus and transactivates genes controlling cell proliferation and simultaneously blocks apoptosis. In contrast, phosphorylation of FOXO3A degrades this apoptotic and antiproliferative transcription factor, achieving the same outcome. Treatments with inhibitors of erbB2 signaling, such as trastuzumab, and NF- κ B signaling with NBD or PS1145 reverse these events, reinstate apoptosis, and block cell proliferation. Blocking degradation of phosphorylated I κ B and phosphorylated FOXO3A by proteasome inhibition with PS341 also returns cells to proapoptotic and reduced proliferative states.

both proliferative and antiapoptotic signals. Furthermore, IKK phosphorylates at least three important transcription factors, including NF- κ B, FOXO3A, and the estrogen receptor (23, 33–35). Previous studies have shown high-level activation of NF- κ B in specific subtypes of breast cancer, particularly those tumors that express erbB2 and are ER negative (6). Human breast cancers that express erbB1 (EGF receptor 1), exemplified by MDA-MB231 and MDA-MB486 cells, may also be equally dependent on competent NF- κ B (12). Although erbB1 is infrequently amplified in human breast cancer, it may be overexpressed in a significant fraction of ER-negative cancers, particularly the erbB2-negative and ER-negative basal-like tumor subtype (36–38). Substantial evidence supports transmission of signals from erbB family receptors through phosphatidylinositol 3-kinase and its downstream pathway (33, 36–38). Eventually, IKK is engaged and the activation of this enzyme leads to NF- κ B activation and release and

degrades FOXO3A, thereby affecting cell proliferation, immunity from apoptosis, and tumor growth.

Application of NF- κ B activation as a therapeutic target to clinical practice seems logical. Therapies that include NF- κ B-directed drugs in combination with erbB1- or erbB2-targeted agents may be used for breast cancers that are either erbB2 positive or harbor overexpressed erbB1. Several new agents are entering clinical practice targeting either erbB1 or erbB2 as well as dual inhibitors that target both. These new small-molecule inhibitors of the erbB2 receptor tyrosine kinase are in phase I and II clinical trials (39). The only NF- κ B inhibitor approved for a cancer indication is bortezomib, a proteasome inhibitor that prevents degradation of I κ B and restrains NF- κ B subunits from entering the nucleus. Newer inhibitors of IKK under development for inflammatory diseases and cancer should be more specific for NF- κ B activation (40). Our results suggest synergistic inhibition of cell proliferation when

trastuzumab is combined with PS1145 or NBD, which disables IKK with bortezomib. Clinical testing of NF- κ B inhibitors in breast cancer, alone or in combination, is appropriate for patients with tumors harboring active

NF- κ B and may be particularly beneficial for patients with erbB1- or erbB2-positive breast cancer.

In support of the essential role of NF- κ B in breast cancer, the current study has shown the following: (a) imposing a

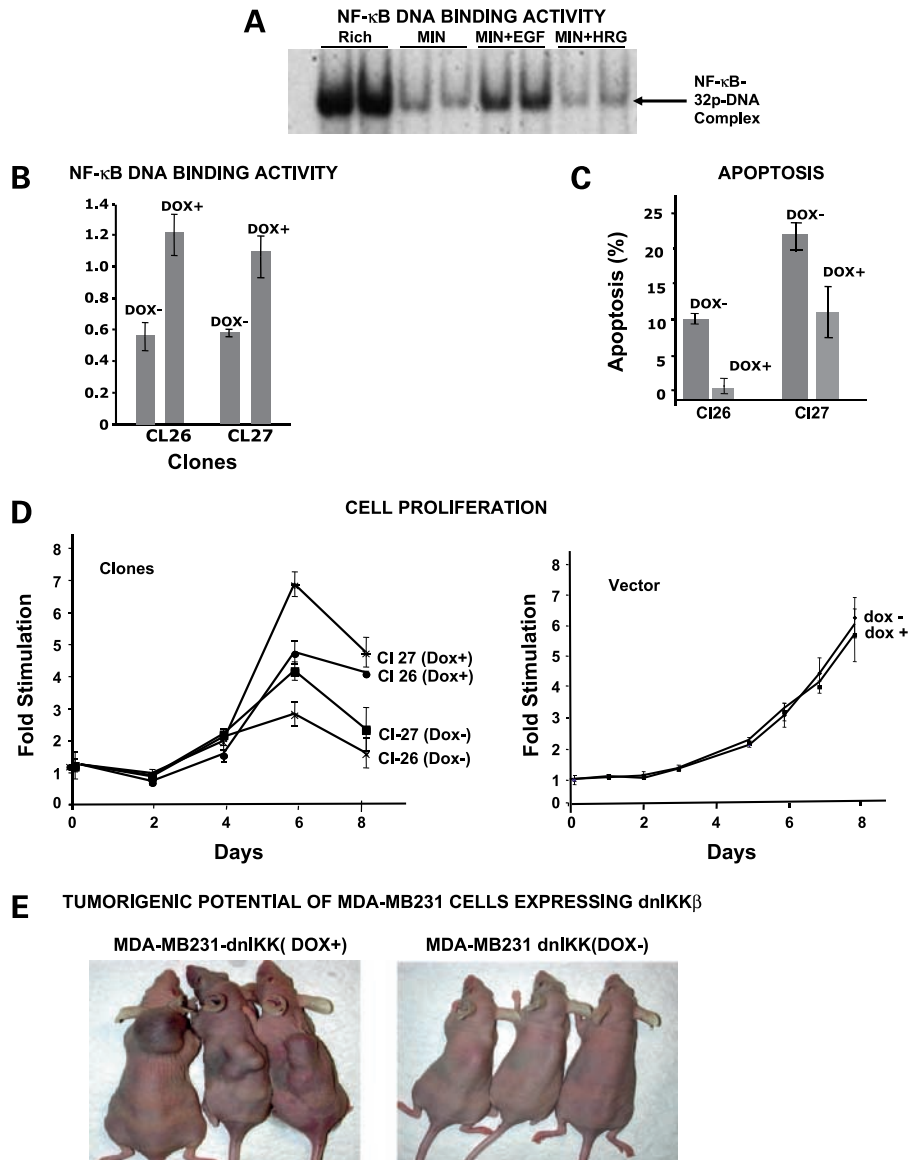


Figure 5. Blocking NF- κ B activation in MDA-MB231 cells and xenograft tumors. **A**, regulation of NF- κ B in MDA-MB231 cells. Nuclear extracts from cells grown in rich medium, minimal medium, minimal medium + EGF (12.5 ng/mL), and minimal medium + HRG (2 nmol/L) were prepared and NF- κ B DNA-binding activity was determined by EMSA as described in Materials and Methods (6). **B**, NF- κ B DNA binding. MDA-MB231 cells were stably transformed by an inducible dnIKK β expression plasmid as described in the legends of Supplementary Fig. S1.⁵ Two stable clones, clone 26 (CL26) and clone 27 (CL27), were selected. These clones were grown and maintained in the presence of DOX (DOX+; 0.5 μ g/mL) in rich medium that does not permit expression of dnIKK β . However, the expression of dnIKK β is turned on on withdrawal of DOX from the growth medium (DOX-). Nuclear extracts from clones 26 and 27 were prepared from cells grown in the presence or absence of DOX and NF- κ B DNA-binding activity was measured by EMSA and quantified by scanning densitometry. The results are reported as band intensity (arbitrary numbers). **C**, apoptosis. Percentage of apoptotic cells in clones 26 and 27 was determined by Annexin V – binding assay (6). Clones 26 and 27 were grown in the presence or absence of DOX as in **B**. **D**, cell proliferation. Proliferation of clones 26 and 27 in the presence or absence of DOX was measured by determining the number of viable cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (41). The results are expressed as fold stimulation of growth at the indicated intervals of time relative to day 0. **E**, xenograft growth of MDA-MB231 cells expressing a DOX-regulated dnIKK β allele. Xenograft tumor growth was studied by implanting 2×10^6 to 3×10^6 of clone 26 cells under the dorsal skin of *nu/nu* mice and measuring tumor growth in the presence or absence of DOX in the drinking water. DOX⁻ animals received just the 5% sucrose solution for drinking. Tumors were photographed at 7 wks for both DOX⁺ and DOX⁻ groups. No xenograft tumors were detected with cells expressing the dnIKK β allele (DOX⁻) even after 30 wks. Results of one of two experiments with indicated number of animals in each group (Supplementary Table S1).⁵

genetic block of NF- κ B activation by inducing dnIKK blocked xenograft growth of an otherwise strongly tumorigenic cell line (MDA-MB231); (b) specific inhibitors of IKK, including the NBD peptide and PS1145, block HRG-induced NF- κ B activation and stop proliferation of erbB2-positive cells in culture; and (c) activated IKK confers to the cells the enhanced cell proliferation and antiapoptotic phenotypes by engaging at least two functionally opposite transcription factors, NF- κ B and FOXO3A. Thus, the members of the NF- κ B activation cascade are participants in the tumorigenesis process in a subclass of breast cancer and are potential targets for therapy tailored for tumors with overactive NF- κ B.

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