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Constitutive activation of nuclear factor-κB is preferentially involved in the proliferation of basal-like subtype breast cancer cell lines

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Constitutive nuclear factor (NF)-κB activation is thought to be involved in survival, invasion, and metastasis in various types of cancers. However, neither the subtypes of breast cancer cells with constitutive NF-κB activation nor the molecular mechanisms leading to its constitutive activation have been clearly defined. Here, we quantitatively analyzed basal NF-κB activity in 35 human breast cancer cell lines and found that most of the cell lines with high constitutive NF-κB activation were categorized in the estrogen receptor negative, progesterone receptor negative, ERBB2 negative basal-like subtype, which is the most malignant form of breast cancer. Inhibition of constitutive NF-κB activation by expression of IκBα super-repressor reduced proliferation of the basal-like subtype cell lines. Expression levels of mRNA encoding NF-κB-inducing kinase (NIK) were elevated in several breast cancer cell lines, and RNA interference-mediated knockdown of NIK reduced NF-κB activation in a subset of the basal-like subtype cell lines with upregulated *NIK* expression. Taken together, these results suggest that constitutive NF-κB activation, partially dependent on NIK, is preferentially involved in proliferation of basal-like subtype breast cancer cells and may be a useful therapeutic target for this subtype of cancer. (*Cancer Sci* 2009; 100: 1668–1674)

Breast cancer is a disease of the mammary epithelium, which is composed of two major types of differentiated cells: luminal epithelial cells and basal or myoepithelial cells.[1] Recent studies have identified self-renewing pluripotent stem cells in mammary epithelium and suggest a model in which these stem cells could differentiate into the luminal- or basal-restricted lineages. Molecular taxonomic analyses of breast cancers by gene expression profiling have identified five breast cancer subtypes: luminal A, luminal B, basal-like, ERBB2-positive, and normal breast-like.[2] This classification is closely associated with the differentiation model of mammary epithelium. Luminal- and basal-like breast cancer subtypes express genes characteristic of the two distinct types of epithelial cells. These subtypes show different clinical courses and responses to therapeutic agents. The basal-like subtype has been associated with aggressive behavior and poor prognosis and typically does not express estrogen receptor (ER), progesterone receptor (PR), or ERBB2 ("triple-negative" phenotype).[3] Therefore, patients with basal-like subtype are unlikely to benefit from currently available targeted therapeutic strategies, such as hormone therapy and Herceptin (Roche, Basel, Switzerland). It is thus crucial to identify effective molecular targets for this subtype of breast cancer.

Nuclear factor (NF)-κB transcription factors are important regulators of the genes necessary for innate and adaptive immune responses and for the survival and proliferation of certain cell types. The NF-κB family is composed of five different proteins, including RelA, RelB, c-Rel, and the precursor and processed products of the *NFKB1* (p105/p50) and *NFKB2* (p100/p52) genes. These proteins homodimerize and/or heterodimerize to form active transcription factors. Two distinct NF-κB pathways have been proposed: the classical pathway, which activates the RelA–p50 complex, and the alternative pathway, which activates the RelB–p52 complex.[4] In normal cells, activation of the classical and alternative pathways is tightly regulated by inhibitor of NF-κB (IκB) family proteins and a p100 protein, respectively. Both NF-κB pathways are aberrantly activated and involved in tumor development in various cancers, including breast cancer.[5,6] Previous studies have revealed that hormone-independent breast cancer cells exhibit constitutive NF-κB activation[7,8] and that the *IKBKE* gene, which encodes a kinase involved in NF-κB activation,[9] is amplified in several breast cancer cell lines.[10] However, the subtypes of breast cancers that show constitutive activation of NF-κB have not yet been clearly defined, and the molecular mechanisms leading to constitutive NF-κB activation in breast cancer cells are not fully understood.

Materials and Methods

Cell culture, transfection, and adenovirus production. The source of cell lines and culture conditions was described previously.[11] MG-132 and SC-514 were purchased from Calbiochem (San Diego, CA, USA). SiRNA for NF-κB-inducing kinase (NIK) (5′-GCCAGUCCGAGAGUCUUGAUCAGAU-3′) and control siRNA were purchased from Invitrogen (Carlsbad, CA, USA). Plasmid vector or siRNA was transfected into cells with Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. Methods of preparation and infection of recombinant adenoviruses were as described previously.[12]

Nuclear extract preparation and EMSA. Cells were harvested at 24 h after medium change, and nuclear extracts were prepared. Methods for nuclear extract preparation and EMSA were described previously.[13] For quantitation, exposed imaging plates were analyzed with a BAS2000 Biomage analyzer (Fujifilm, Tokyo, Japan). For supershift analysis, extracts were preincubated for 30 min with antibodies described below. Anti-Omni probe antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a control.

Western blot analysis. Methods for western blot analysis were described previously.[13] The primary antibodies used were anti-RelA, anti-RelB, and anti-p50 (Santa Cruz Biotechnology), anti-α-tubulin (Calbiochem), anti-NIK and anti-Cyclin D1 (Cell Signaling Technologies, Beverly, MA, USA), and anti-p52 (Millipore, Bedford, MA, USA). All secondary antibodies were purchased from GE Healthcare (Piscateway, NJ, USA).

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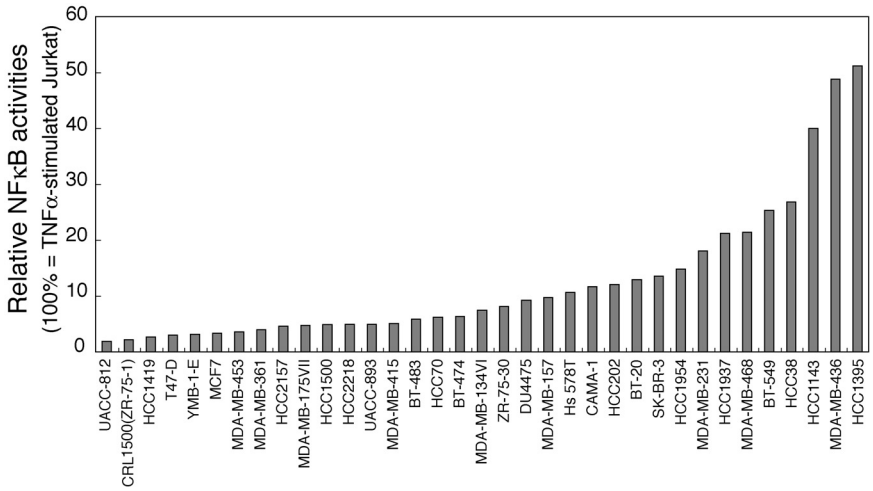

Fig. 1. Basal nuclear factor (NF)-κB levels in 35 human breast cancer cell lines determined by EMSA. The data are shown as a ratio (%) relative to the NF-κB level of tumor necrosis factor (TNF)α-stimulated Jurkat ($n = 3$).

Quantitative real-time PCR analysis for NIK mRNA expression. Methods for RNA isolation and quantitative real-time PCR were described previously.[13] The primer/probe set for the human *NIK* (*MAP3K14*) gene (Hs00177695_m1) and the *β-actin* gene (4326315E) were purchased from Applied Biosystems (Foster City, CA, USA). The *β-actin* gene was used as an internal reference control, and *NIK* mRNA level was estimated using the comparative threshold cycle method.

Proliferation assay. Cells were infected with adenoviral vector as described previously and plated at 5×10^5 cells per well in a 24-well plate (day 0). On days 3 and 5 after adenoviral infection, cells were trypsinized, and the viable cell number was counted using the Trypan Blue exclusion method. Methods for flow cytometric analysis were described previously.[13]

Results

Constitutive NF-κB activation in basal-like subtype breast cancer cell lines. To identify subtypes of breast cancer cells with constitutively activated NF-κB, we first analyzed the basal NF-κB DNA binding activities in 35 human breast cancer cell lines with EMSA. For quantitative analysis, the intensity of retarded bands corresponding to the NF-κB and DNA complex was measured by an image analyzer, and the NF-κB level of each cell line was normalized to that of tumor necrosis factor (TNF)α-stimulated Jurkat cells. These experiments were done in triplicate, and means are presented in Figure 1. The results of EMSA for the eight cell lines with the highest level of NF-κB activity and two control cell lines are shown in Figure 2(a). Subtypes of various cell lines and their basal NF-κB activity are summarized in Table 1.[14,15] Interestingly, most of the cell lines with highly elevated NF-κB activities were categorized into the basal-like subtype. In contrast, most of the cell lines with low NF-κB activities were categorized into the luminal subtype. To determine the composition of constitutively activated NF-κB subcomponents in the eight cell lines, we carried out supershift EMSA. Constitutively activated NF-κB complexes were found to contain RelA, RelB, p50, and p52 in all cell lines except for MDA-MB-468 (Fig. 2b). Taken together, these results suggest that most of the basal-like subtype breast cancer cell lines exhibit constitutive activation of both the classical and the alternative NF-κB pathways.

Inhibition of constitutive NF-κB activation suppressed proliferation of the basal-like subtype breast cancer cell lines. To determine the functional significance of constitutive NF-κB activation, we examined the effects of adenovirus-mediated expression of the non-degradable IκBα super-repressor (IκBαSR), which has alanine substitutions at serines 32 and 36. Adenoviral infection efficiency was tested in three cell lines (MDA-MB-436, HCC1143, and MDA-MB-468) using an adenovirus expressing GFP (Adeno-GFP), and the infection efficiencies were nearly 90% in all cell lines tested (MOI 5, data not shown). Expression of IκBαSR protein was confirmed by western blot analysis 2 days after infection (Fig. 3a). In adenovirus-infected cells expressing IκBαSR (Adeno-IκBαSR),

Table 1. List of basal nuclear factor (NF)-κB activities and gene clusters in 35 breast cancer cell lines

Cell line	NF-κB activity (%)	Gene cluster
HCC1395	51.2	ND
MDA-MB-436	48.8	Basal
HCC1143	40.0	Basal
HCC38	26.8	Basal
BT-549	25.3	Basal
MDA-MB-468	21.4	Basal
HCC1937	21.2	Basal
MDA-MB-231	18.1	Basal/mesenchymal
HCC1954	14.9	Basal
SK-BR-3	13.6	Luminal
BT-20	13.0	Basal
HCC202	12.1	Luminal
CAMA-1	11.7	Luminal
Hs 578T	10.7	Basal
MDA-MB-157	9.8	Basal/mesenchymal
DU4475	9.3	ND
ZR-75-30	8.2	Luminal
MDA-MB-134VI	7.5	Luminal
BT-474	6.4	Luminal
HCC70	6.2	Basal
BT-483	5.9	Luminal
MDA-MB-415	5.1	Luminal
UACC-893	5.0	ND
HCC2218	5.0	ND
HCC1500	4.9	Basal/luminal
MDA-MB-175VII	4.8	Luminal
HCC2157	4.6	Basal
MDA-MB-361	4.0	Luminal
MDA-MB-453	3.6	Luminal
MCF7	3.4	Luminal
YMB-1-E	3.2	ND
T47-D	3.0	Luminal
HCC1419	2.7	ND
CRL1500 (ZR-75-1)	2.2	Luminal
UACC-812	1.9	Luminal

ND, not determined.

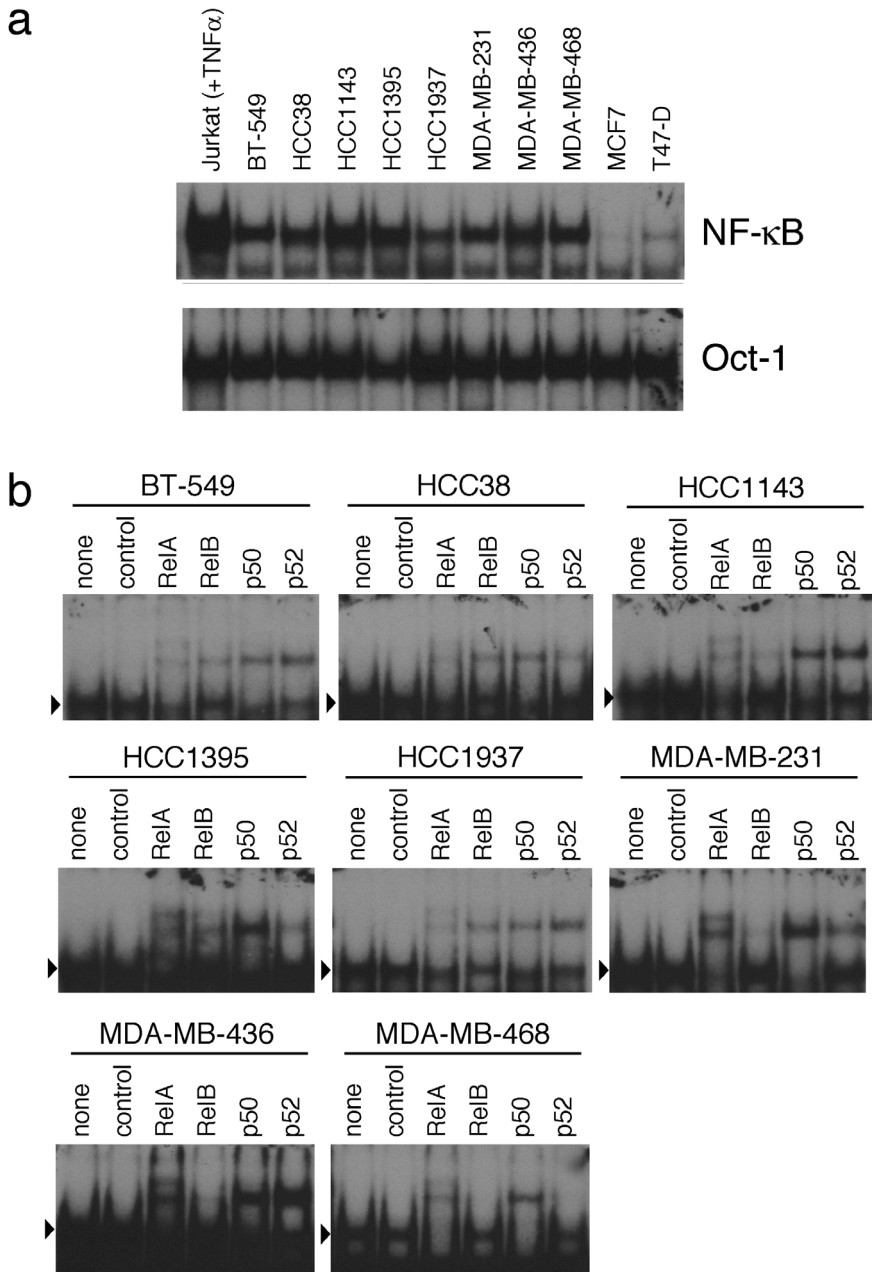

Fig. 2. (a) EMSA for nuclear factor (NF)-κB and octamer transcription factor (Oct)-1 with nuclear extracts from the eight cell lines with the highest level of NF-κB activity, two control cell lines, and TNFα-stimulated Jurkat. (b) Supershift EMSA with anti-RelA, anti-RelB, anti-p50, or anti-52 antibody or control antibody. The positions of unshifted complexes are shown by arrowheads.

a band corresponding to full-length IκBαSR and a fast-migrating band were detected. Because the second ATG codon of the gene encoding IκBαSR is located within a weak Kozak sequence (gcc-ATG-gag), we speculated that this band might correspond to an N-terminal truncated form of IκBαSR.

The activity of NF-κB, measured by EMSA, was nearly abolished at 3 and 5 days after infection with Adeno-IκBαSR, but was not significantly changed in Adeno-GFP-infected cells (Fig. 3b). Activity of the control transcription factor, octamer transcription factor (Oct)-1, was not affected by infection with either virus. These results indicate that Adeno-IκBαSR causes efficient and specific suppression of NF-κB activation in these cells. Next, we analyzed the effects of NF-κB suppression on cell growth (Fig. 3c). Although the growth rate of cells infected with Adeno-GFP was slightly reduced compared with that of uninfected cell lines, cells infected with Adeno-IκBαSR showed a dramatic reduction in proliferation in all cell lines analyzed. To investigate whether the growth inhibitory effects of Adeno-IκBαSR infection are related to the induction of apoptosis, the effect of Adeno-IκBαSR infection on apoptosis of MDA-MB-436 was examined with DNA and propidium iodide (PI) staining at 5 days after viral infection. Whereas uninfected cells and Adeno-GFP-infected cells showed a low basal sub-G_1 cell population (3.1 and 5.6%, respectively), Adeno-IκBαSR-infected cells showed an increased proportion of sub-G_1 cells (19.7%; Fig. 3d). These data suggest that Adeno-IκBαSR infection induces apoptosis of basal-like subtype breast cancer cell lines. However, Adeno-IκBαSR-induced inhibition of proliferation was very severe in MDA-MB-436 (Fig. 3c), and the G_1/G_2 ratio was reduced in Adeno-IκBαSR-infected cells (Fig. 3d). Thus, constitutive NF-κB activation may also be involved in cell cycle progression in basal-like subtype breast cancer cells.

Involvement of NIK in constitutive NF-κB activation in basal-like subtype breast cancer cell lines. NIK, a protein kinase pivotal in the activation of the alternative NF-κB pathway, has recently been reported to be involved in aberrant NF-κB activation in hematological and solid tumors.[16–19] These observations led us to

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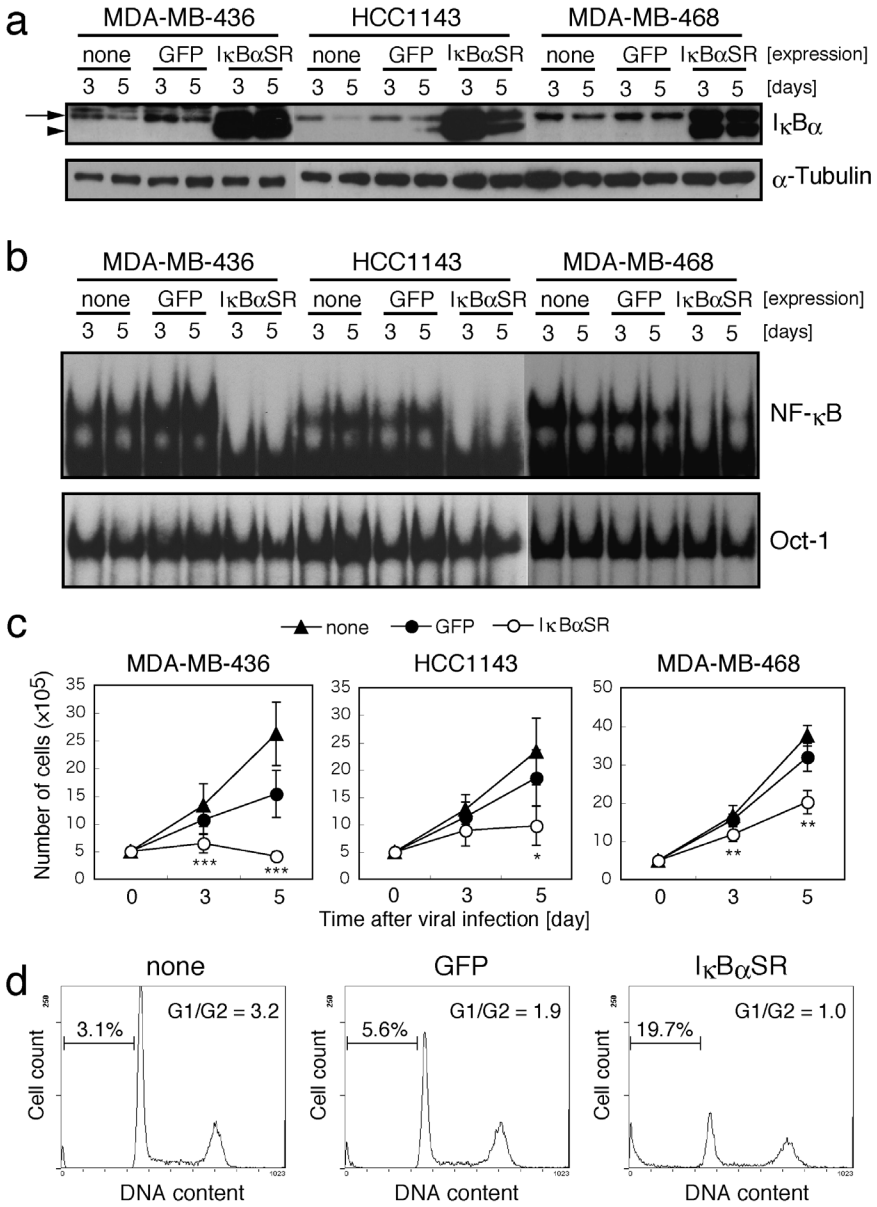

Fig. 3. Reduced proliferation in cell lines with constitutive nuclear factor (NF)-κB activation by inhibition of NF-κB. (a) Western blot analysis of IκBα in three cell lines at 3 or 5 days after Adeno-GFP or Adeno-IκBαSR infection. The arrow indicates endogenous IκBα or IκBαSR, and the arrowhead indicates a putative truncated IκBαSR lacking the sequences upstream of the second ATG. Expression of α-tubulin is shown as a loading control. (b) EMSA in three cell lines at 3 and 5 days after Adeno-GFP or Adeno-IκBαSR infection. The Oct-1 probe is shown as a loading control. (c) Proliferation analysis of three untreated, Adeno-GFP-infected, or Adeno-IκBαSR-infected cell lines. Experiments were done in triplicate, and the standard deviation is indicated on the plots. $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$ relative to Adeno-GFP-infected samples. (d) MDA-MB-436 was infected with Adeno-IκBαSR or Adeno-GFP and harvested 5 days after viral infection. Cell nuclei were stained with propidium iodide, and cellular DNA contents were measured with a flow cytometer.

hypothesize that NIK is also involved in the constitutive activation of NF-κB in breast cancer cell lines. To test this hypothesis, we compared the pattern of basal NF-κB activity and *NIK* mRNA expression in the 35 breast cancer cell lines using our recently reported gene expression profiles.[11] As shown in Figure 4(a), basal NF-κB activities had a weak positive correlation with *NIK* mRNA expression levels ($r = 0.51$). To further characterize this correlation, we measured the *NIK* mRNA levels in the eight cell lines with the highest level of NF-κB activity and two control cell lines (MCF7 and T47-D) by quantitative real-time PCR. Four of the eight cell lines exhibited upregulation of *NIK* mRNA compared with controls (Fig. 4b).

We then examined NIK protein levels in the cell lines by western blot analysis. However, NIK protein was not detected in cells cultured under normal conditions. Previous studies demonstrated that NIK protein is maintained at low levels by proteasome-mediated degradation in most cells and that stimulation-dependent stabilization of NIK protein results in activation of the alternative NF-κB pathway.[20] We therefore examined NIK expression in the presence of a proteasome inhibitor, MG-132. As shown in Figure 4(c), NIK protein levels were significantly increased by treatment with MG-132 in all of the cell lines with constitutive NF-κB activation, with the exception of HCC1395 and MDA-MB-468. In contrast, NIK protein was not detected in the control cell line MCF7, even in the presence of MG-132. These results suggest that NIK expression levels are preferentially upregulated in the basal-like subtype breast cancer cell lines with constitutive NF-κB activation.

In order to determine whether NIK contributes to constitutive NF-κB activation, RNA interference (RNAi)-mediated knockdown of NIK was carried out in the six cell lines with high levels of NIK. To confirm depletion of NIK protein, we carried out western blot analysis with lysates from NIK- or mock-siRNA-transfected cell lines with or without treatment of MG-132. Although NIK protein levels were increased with MG-132 treatment in the cells transfected with mock siRNA, NIK protein was expressed at extremely low or barely detectable levels in NIK siRNA-transfected cells even after MG132 treatment (Fig. 5a). EMSA were then carried out to evaluate the effect of NIK RNAi on NF-κB activity. Knockdown of NIK caused an apparent reduction of constitutive NF-κB activation in three of six cell lines (BT-549, HCC1143, and HCC1937) and a slight reduction in the rest of them (Fig. 5b). Western

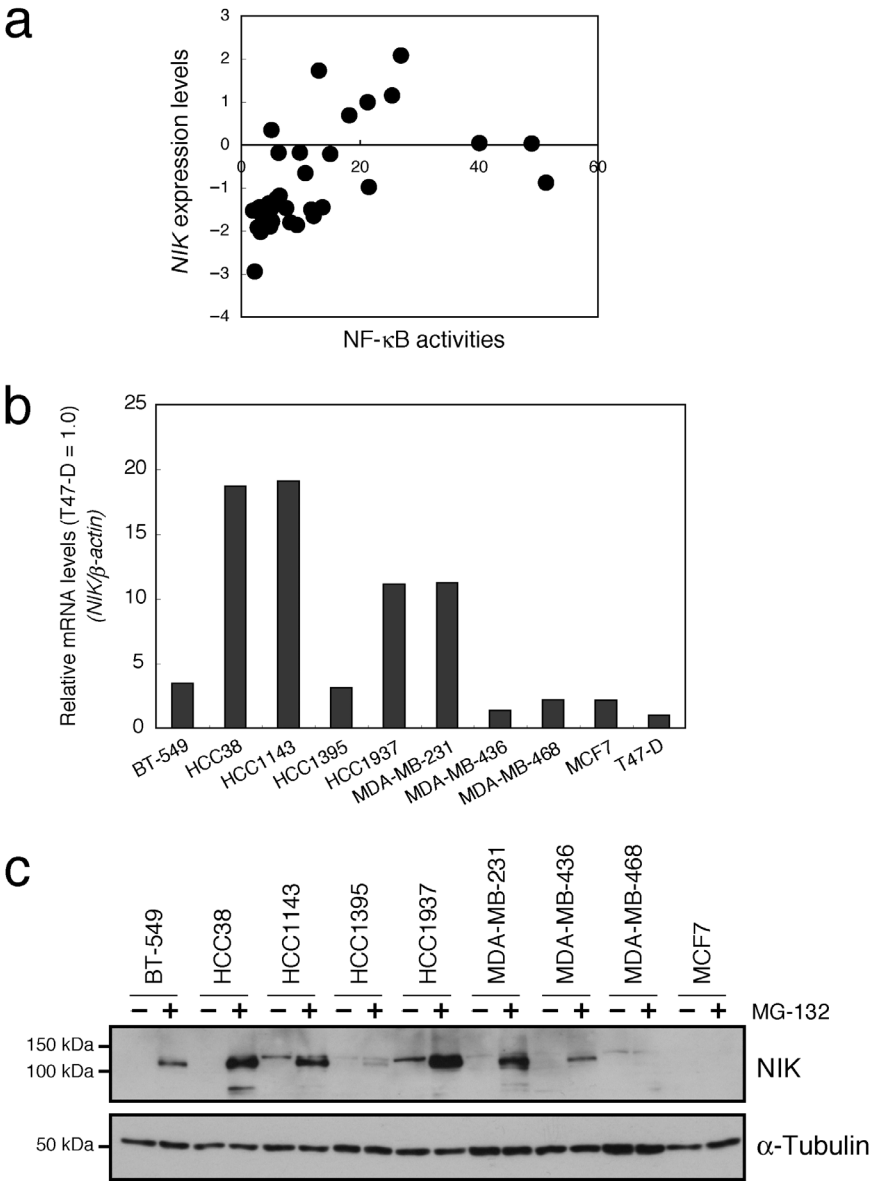

Fig. 4. (a) Scatter plot of basal nuclear factor (NF)-κB activities and NF-κB-inducing kinase (NIK) mRNA levels in the 35 breast cancer cell lines. (b) Real-time PCR analysis of NIK mRNA levels in ten cell lines. β-Actin mRNA was used as an internal reference control. The data for each cell line are shown as a ratio relative to that of control T-47D. (c) Western blot analysis of NIK in nine cell lines cultured in the presence or absence of the proteasomal inhibitor MG-132 (20 μM) for 3 h. Expression of α-tubulin is shown as a loading control.

blot analysis was carried out with the nuclear extracts used for EMSA. Protein levels of both RelB and p52, but not RelA, were reduced in the NIK siRNA-transfected nuclear extracts from the three cell lines (BT-549, HCC1143, and HCC1937) that showed an apparent reduction in the NF-κB activation in the EMSA (Fig. 5c). These data suggest that knock down of NIK predominantly inhibited constitutive activation of the alternative NF-κB pathway by suppressing nuclear translocation of the RelB–p52 complex. In contrast, the reduction in RelB and p52 protein levels was very weak in the NIK siRNA-transfected nuclear extracts from the other three cell lines. Although the precise reason for this observation is not clear, we speculate that the effect of RNAi was insufficient to completely inhibit the function of NIK in these cell lines.

Discussion

Our present data show that NF-κB is constitutively activated in ER^-, PR^-, and $ERBB2^-$ basal-like subtype breast cancer cells. Recent studies have suggested a model in which the basal-like subtype and luminal subtype breast cancers are distinguished by their cells of origin: these two subtypes arise from basal epithelial cells and luminal epithelial cells, respectively.[2] Mutant mice with a defect in the NF-κB pathway, such as NIK, IκB kinase (IKK) α, or receptor activator of NF-κB (RANK), show abnormalities in mammary development.[21–23] Because such mutant mice show a partial block in mammary gland development, the NF-κB pathway may be preferentially involved in the development of a subtype of mammary epithelium. In mammary epithelial cells, knockdown of breast cancer (BRCA)1, which is a positive regulator of differentiation of luminal epithelial cells,[24] caused a reduction in expression of the TNF receptor associated factor *(TRAF)3* gene.[25] Because TRAF3 acts as a negative regulator of NF-κB,[20] these reports suggest that BRCA1-mediated suppression of NF-κB may be required for differentiation of luminal epithelial cells. Thus, NF-κB may have an important role in the development of basal epithelial cells but not in that of luminal epithelial cells. Based on these observations, we speculated that NF-κB would be preferentially involved in tumorigenesis of basal epithelial cells. Because basal epithelial cells do not express the hormone receptors,[26] basal-like subtype breast cancer cells may lack the expression of these receptors. NF-κB has a key role in ERBB2-induced mammary tumorigenesis,[27,28] suggesting that the constitutive activation of NF-κB in basal-like subtype may play

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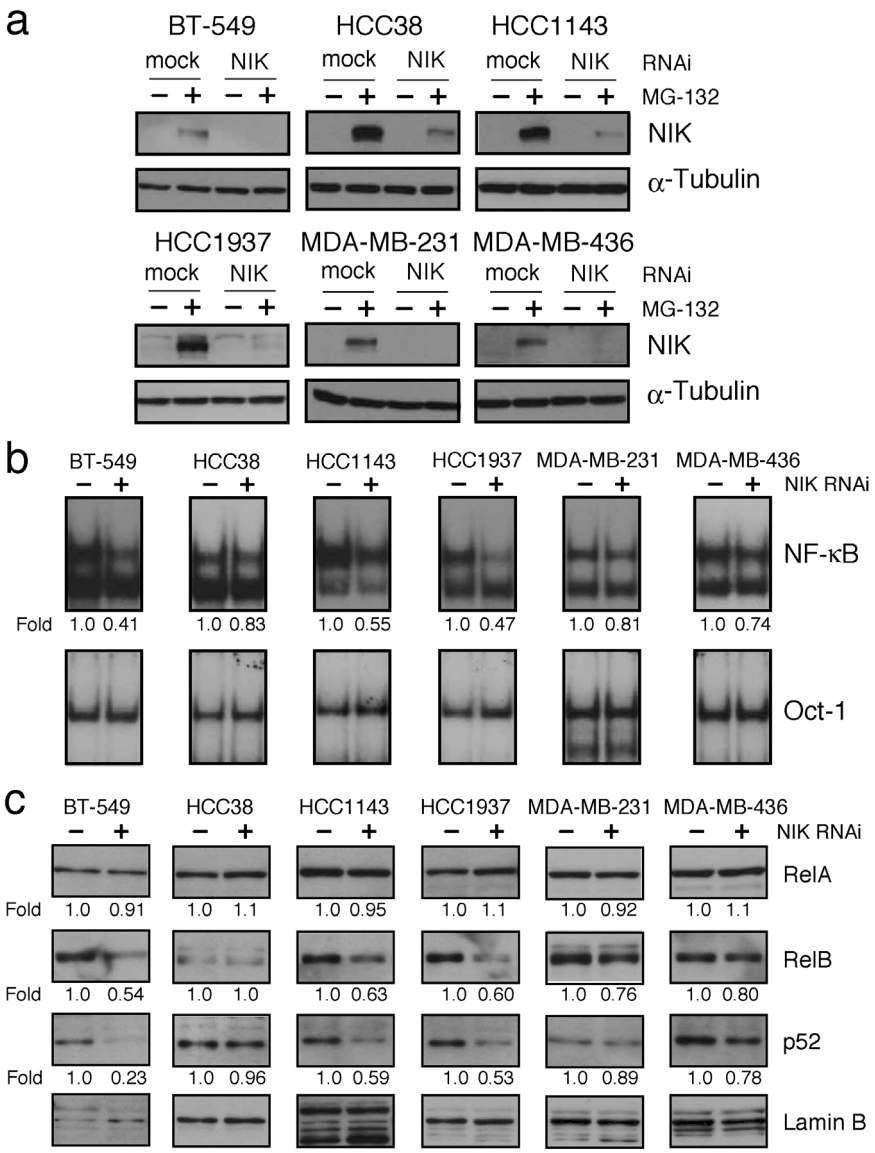

Fig. 5. (a) Western blot analysis of NF-κB-inducing kinase (NIK) in six cell lines with or without treatment of MG-132 (3 h) at 3 days after siRNA transfection. (b) EMSA for NF-κB with nuclear extracts of the six cell lines at 3 days after siRNA transfection. Oct-1 probe is shown as a loading control. The intensity of bands of NF-κB–DNA complexes was normalized to that of Oct-1–DNA complexes, and the values of fold changes are indicated. (c) Western blot analysis of RelA, RelB, and p52 in nuclear extracts from the six cell lines with upregulated NIK expression levels 3 days after NIK or mock-siRNA transfection. Lamin B is shown as a loading control. The intensities of RelA, RelB, and p52 bands were normalized to that of Lamin B, and the values of fold changes are indicated.

a role similar to that of ERBB2 in tumorigenesis of the luminal subtype. We think that these observations may explain why ER$^-$, PR$^-$, and ERBB2$^-$ basal-like subtype breast cancer cells exhibit high constitutive NF-κB activation.

In the present study, knockdown of NIK resulted in a reduction of constitutive NF-κB activation in six of eight cell lines with high constitutive NF-κB activation in EMSA, suggesting that NIK is involved in constitutive NF-κB activation in breast cancer cells (Fig. 5b). Recently, deregulation of NIK activity and its involvement in tumorigenesis have been reported in various cancers, including multiple myeloma,[16,17] melanoma,[18] adult T-cell lymphoma,[19] and Hodgkin's lymphoma.[19] In multiple myeloma, several genetic aberrations that cause upregulation of NIK activity have been identified, such as *NIK* gene amplification or translocation and loss of function mutations in genes encoding negative regulators of NIK (TRAF2, TRAF3, cellular inhibitor of apoptosis protein (c-IAP)1, or c-IAP2). In melanoma, the lymphotoxin-β receptor was suggested to induce constitutive NIK activation. In adult T-cell lymphoma and Hodgkin's lymphoma, upregulation of *NIK* mRNA expression was shown to cause aberrant NIK activation. Forced expression of NIK induced transformation of rat fibroblasts through the upregulation of NF-κB activity,[19] suggesting that deregulation and aberrant activation of the NIK–NF-κB pathway accelerates tumorigenesis. In our data, four of six cell lines, in which constitutive NF-κB activation was reduced by knockdown of NIK, showed upregulation of *NIK* mRNA, suggesting that increased levels of *NIK* mRNA expression may be one of the major causes of the upregulation of NIK activity in breast cancer cells (Fig. 4b). Given that some of the cell lines with constitutive NF-κB activation also had low levels of *NIK* mRNA (BT-549 and MDA-MB-436), NIK activation may be controlled by mechanisms other than expression of mRNA. Furthermore, our data showed that NIK RNAi failed to suppress constitutive activation of the classical NF-κB pathway in the breast cancer cell lines, suggesting that constitutive activation of the classical pathway is induced in a NIK-independent manner (Fig. 5c). Recent studies have shown that RIP1 is constitutively polyubiquitinated in several cancer cell lines, including MDA-MB-231.[29] Because polyubiquitination of RIP1 is a crucial process in activation of the classical NF-κB pathway,[30,31] receptor-interacting protein (RIP)1 may be involved in constitutive activation of the classical NF-κB pathway in breast cancer cell lines. Related to this, we have examined the effects of an IKK inhibitor, SC-514, on constitutive NF-κB activation in the eight breast cancer cell lines we used. Although the cells were treated with sufficient SC-514 (100 μM, 24 h),[32] the SC-514 treatment induced only a

partial reduction in constitutive NF-κB activation in four of the eight (HCC1143, HCC1395, MDA-MB-231, and MDA-Mb-436: data not shown). These data suggest that constitutive NF-κB activation is induced in part by an IKK-independent mechanism. Very recently, the signal transducers and activators of transcription (STAT)3 pathway has been reported to induce constitutive NF-κB activation in an IKK-independent manner.[33] Thus, this STAT3 pathway represents one additional candidate mechanism for inducing constitutive NF-κB activation in breast cancer cells.

Because *Cyclin D1* was proposed as a pivotal NF-κB target gene in mammary development,[23] we examined whether *Cyclin D1* is a target of NF-κB in breast cancer cell lines by western blot analysis. However, Cyclin D1 expression was not affected by Adeno-IκBαSR infection (data not shown). Furthermore, our data of flow cytometric analysis showed that the G_1 population was reduced rather than increased in Adeno-IκBαSR-infected cells (Fig. 3d). These data suggest that *Cyclin D1* is not a major target gene of constitutively activated NF-κB in breast cancer cell lines.

Although basal-like subtype breast cancer was originally identified as having properties of mammary basal epithelial cells, recent studies have revealed that these tumor cells also have stem cell-like characteristics.[34,35] Thus, the NF-κB pathway may be involved in the maintenance or generation of stem cell-like properties in breast cancer cells.

In conclusion, the transcription factor NF-κB is constitutively activated in most of the basal-like subtype breast cancer cell lines and is preferentially involved in proliferation of these cells. The findings presented here suggest that the NF-κB signaling axis may be a therapeutic target for basal-like subtype breast cancer.

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References

1 Smalley M, Ashworth A. Stem cells and breast cancer: a field in transit. *Nat Rev Cancer* 2003; **3**: 832–44.
2 Vargo-Gogola T, Rosen JM. Modelling breast cancer: one size does not fit all. *Nat Rev Cancer* 2007; **7**: 659–72.
3 Rakha EA, Reis-Filho JS, Ellis IO. Basal-like breast cancer: a critical review. *J Clin Oncol* 2008; **26**: 2568–81.
4 Hayden MS, Ghosh S. Signaling to NF-κB. *Genes Dev* 2004; **18**: 2195–224.
5 Karin M. Nuclear factor-κB in cancer development and progression. *Nature* 2006; **441**: 431–6.
6 Inoue J, Gohda J, Akiyama T *et al*. NF-κB activation in development and progression of cancer. *Cancer Sci* 2007; **98**: 268–74.
7 Nakshatri H, Bhat-Nakshatri P, Martin DA *et al*. Constitutive activation of NF-κB during progression of breast cancer to hormone-independent growth. *Mol Cell Biol* 1997; **17**: 3629–39.
8 Biswas DK, Shi Q, Baily S *et al*. NF-κB activation in human breast cancer specimens and its role in cell proliferation and apoptosis. *Proc Natl Acad Sci USA* 2004; **101**: 10137–42.
9 Peters RT, Liao SM, Maniatis T. IKKε is part of a novel PMA-inducible IκB kinase complex. *Mol Cell* 2000; **5**: 513–22.
10 Boehm JS, Zhao JJ, Yao J *et al*. Integrative genomic approaches identify IKBKE as a breast cancer oncogene. *Cell* 2007; **129**: 1065–79.
11 Ito E, Honma R, Yanagisawa Y *et al*. Novel clusters of highly expressed genes accompany genomic amplification in breast cancers. *FEBS Lett* 2007; **581**: 3909–14.
12 Horie R, Watanabe T, Morishita Y *et al*. Ligand-independent signaling by overexpressed CD30 drives NF-κB activation in Hodgkin-Reed-Sternberg cells. *Oncogene* 2002; **21**: 2493–503.
13 Yamaguchi N, Oyama T, Ito E *et al*. NOTCH3 signaling pathway plays crucial roles in proliferation of ErbB2-negative human breast cancer cells. *Cancer Res* 2008; **68**: 1881–8.
14 Charafe-Jauffret E, Ginestier C, Monville F *et al*. Gene expression profiling of breast cell lines identifies potential new basal markers. *Oncogene* 2006; **25**: 2273–84.
15 Neve RM, Chin K, Fridlyand J *et al*. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* 2006; **10**: 515–27.
16 Annunziata CM, Davis RE, Demchenko Y *et al*. Frequent engagement of the classical and alternative NF-κB pathways by diverse genetic abnormalities in multiple myeloma. *Cancer Cell* 2007; **12**: 115–30.
17 Keats JJ, Fonseca R, Chesi M *et al*. Promiscuous mutations activate the noncanonical NF-κB pathway in multiple myeloma. *Cancer Cell* 2007; **12**: 131–44.
18 Dhawan P, Su Y, Thu YM *et al*. The lymphotoxin-β receptor is an upstream activator of NF-κB-mediated transcription in melanoma cells. *J Biol Chem* 2008; **283**: 15399–408.
19 Saitoh Y, Yamamoto N, Dewan MZ *et al*. Overexpressed NF-κB-inducing kinase contributes to the tumorigenesis of adult T-cell leukemia and Hodgkin Reed-Sternberg cells. *Blood* 2008; **111**: 5118–29.
20 Liao G, Zhang M, Harhaj EW *et al*. Regulation of the NF-κB-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation. *J Biol Chem* 2004; **279**: 26 243–50.
21 Fata JE, Kong YY, Li J *et al*. The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell* 2000; **103**: 41–50.
22 Nishimura T, Koike R, Miyasaka M. Mammary glands of Aly mice: developmental changes and lactation-related expression of specific proteins, α-casein, GLyCAM-1 and MAdCAM-1. *Am J Reprod Immunol* 2000; **43**: 351–8.
23 Cao Y, Bonizzi G, Seagroves TN *et al*. IKKα provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. *Cell* 2001; **107**: 763–75.
24 Liu S, Ginestier C, Charafe-Jauffret E *et al*. BRCA1 regulates human mammary stem/progenitor cell fate. *Proc Natl Acad Sci USA* 2008; **105**: 1680–5.
25 Furuta S, Jiang X, Gu B *et al*. Depletion of BRCA1 impairs differentiation but enhances proliferation of mammary epithelial cells. *Proc Natl Acad Sci USA* 2005; **102**: 9176–81.
26 Sting J, Caldas C. Molecular heterogeniety of breast carcinomas and the cancer stem hypothesis. *Nat Rev Cancer* 2007; **7**: 791–9.
27 Cao Y, Luo JL, Karin M. IκB kinase α activity is required for self-renewal of ErbB2/Her2-transformed mammary tumor-initiating cells. *Proc Natl Acad Sci USA* 2007; **104**: 15852–7.
28 Liu M, Ju X, Willmarth NE *et al*. Nuclear factor-κB enhances ErbB2-induced mammary tumorigenesis and neoangiogenesis *in vivo*. *Am J Pathol* 2009; **174**: 1910–20.
29 Bertrand MJ, Milutinovic S, Dickson KM *et al*. cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. *Mol Cell* 2008; **30**: 689–700.
30 Mahoney DJ, Cheung HH, Mrad RL *et al*. Both cIAP1 and cIAP2 regulate TNFα-mediated NF-κB activation. *Proc Natl Acad Sci USA* 2008; **105**: 11778–83.
31 Varfolomeev E, Goncharov T, Fedorova AV *et al*. c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFα)-induced NF-κB activation. *J Biol Chem* 2008; **283**: 24295–9.
32 Kishore N, Sommers C, Mathialagan S *et al*. A selective IKK-2 inhibitor blocks NF-κB-dependent gene expression in interleukin-1β-stimulated synovial fibroblasts. *J Biol Chem* 2003; **278**: 32861–71.
33 Lee H, Herrmann A, Deng JH *et al*. Persistently activated Stat3 maintains constitutive NF-κB activity in tumors. *Cancer Cell* 2009; **15**: 283–93.
34 Honeth G, Bendahl PO, Ringner M *et al*. The CD44+/CD24-phenotype is enriched in basal-like breast tumors. *Breast Cancer Res* 2008; **10**: R53.
35 Ben-Porath I, Thomson MW, Carey VJ *et al*. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet* 2008; **40**: 499–507.

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