Nerve Growth Factor Is a Potential Therapeutic Target in Breast Cancer

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Abstract

We show here that nerve growth factor (NGF), the prototypic neurotrophin, can be targeted in breast cancer to inhibit tumor cell proliferation, survival, and metastasis. Analysis of a series of biopsies revealed widespread expression of NGF in the majority of human breast tumors, with anti-NGF immunoreactivity concentrated in the epithelial cancer cells. Moreover, immunodeficient mice xenografted with human breast cancer cells and treated with either anti-NGF antibodies or small interfering RNA against NGF displayed inhibited tumor growth and metastasis. Such treatments directed against NGF induced a decrease in cell proliferation with a concomitant increase in apoptosis of breast cancer cells and an inhibition of tumor angiogenesis. Together, these data indicate that targeting NGF in breast cancer may have therapeutic ramifications. [Cancer Res 2008;68(2):346–51]

Introduction

Despite considerable progress in both the diagnosis and treatment, breast cancer remains the second leading cause of cancer deaths among women. The multifactorial nature of breast carcinogenesis, as well as the cellular and molecular diversity within tumors, have rendered difficult for the development of molecularly targeted treatments that can be applied to the broad range of breast tumors. This is well-illustrated with the proto-oncogenic tyrosine kinase membrane receptor Erb-B2 and its corresponding peptidic drug Herceptin (1); although Herceptin has proven to be a very efficient antibreast cancer drug, the fact that only 20% of breast cancers overexpress Erb-B2 is a limitation to its wider use in therapy. Thus, the identification of more universal molecular markers and targets is clearly needed.

It has previously been shown that nerve growth factor (NGF), the first isolated neurotrophin well-known for its role in nervous system development, is able to stimulate the *in vitro* growth and survival of breast cancer cells through its activation of the tyrosine kinase receptor TrkA and the death receptor $p75^{NTR}$ (2–5). Interestingly, NGF cooperates with p185 (HER2) to activate breast cancer cell growth (2), and the reference drug used in hormonotherapy, Tamoxifen, inhibits the proliferative effects of NGF (3). These *in vitro* effects of NGF on breast cancer cells, and the fact that both NGF receptors (TrkA and $p75^{NTR}$) are expressed in all breast tumors (6–8), suggested that NGF is actively involved in mammary carcinogenesis. An *in vivo* demonstration of this hypothesis, however, has not yet been provided.

In this study, we investigated NGF expression in human breast tumor biopsies and applied anti-NGF treatments to immunodeficient mice xenografted with human breast cancer cells. Our results show ubiquitous NGF expression in the epithelial compartment of the majority of breast tumors as well as decreases in tumor growth and metastasis that were inducible with anti-NGF treatments in an animal model. Together, these results show the effect of NGF on breast cancer cell development *in vivo* and establish the value of this growth factor as a potential therapeutic target.

Materials and Methods

Human breast biopsies. Breast carcinoma specimens were obtained from patients treated by mastectomy at the Center Oscar Lambret and the Clinique du Parc (Lille, France) in 1990, selected on the basis of being the first and unilateral cancer; these samples were collected with institutional safety review board approval and have been characterized previously (9). For each tissue sample, the following clinicopathologic information were obtained: histologic subclassification, tumor values [from the tumor-node-metastasis (TNM) classification of the Unio Internationale Contra Cancrum (UICC); ranging from T_0 to T_4], histologic grade [according to Bloom and Richardson (10)], the axillary lymph node status, and the hormone receptor status (estrogen receptor and progesterone receptor), determined in femtomoles of receptors per milligram of cytosolic protein and considered positive >15 fmol/mg. Furthermore, the age and menopausal status of patients were recorded, and life expectancy was followed up.

Real-time reverse transcription-PCR. After pulverizing breast tumors in nitrogen liquid, total RNA was isolated with tri reagent (Euromedex) and treated with DNase (Invitrogen). Reverse transcription was performed with 1 μg of RNA, 0.5 μg of random hexamers, and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) for 10 min at 25°C, 50 min at 37°C, and 15 min at 70°C in a final volume of 20 μL. Real-time PCR amplifications were performed using a Quantitect SYBRGreen PCR kit (Qiagen) with 2 μL of 1:10 cDNA and 500 nmol/L of primers. The primers used were as follows: 5′-CAACAGGACTCACAGGAGCA-3′ and 5′-ACCTCTCCCAACACCATCAC-3′ for *NGF* transcript, and 5′-GTGATGTG-CAGCTGATCAAGACT-3′ and 5′-GATGACCAGCCCAAAGGAGA-3′ for *RPLP0* (human acidic ribosomal phosphoprotein P0), which was used as a reference gene. The subsequent PCR conditions were 40 cycles and were carried out in the following manner: 95°C for 15 s, 60°C for 20 s, and 72°C for 30 s. Data were analyzed using the MX4000 PCR system software (Stratagene).

siRNA against NGF. Double strand siRNA oligonucleotides targeting *NGF*, siRNA 1 5′-GACCACCGCCACAGACAUUTT-3′ and 3′-TTCUG-GUGGCGGUGUCUGUAG-5′, siRNA 2 5′-GGCAAGGAGGUGAUGGUGTT-3′ and 3′-TTCCCGUUCCUCCACUACCAC-5′, were designed based on the published sequence of *NGF* (accession number NM_002506) and synthesized by Eurogentec. One pair of negative control siRNA were designed,

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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with sequences different from siRNA-*NGF*: 5′-*GCUGACCCUGAAGUUCAUCTT*-3′ and 3′-*TTCGACUGGGACUUCAAGUAG*-5′. This control siRNA was eventually labeled with FITC at the 3′ end for transfection rate evaluation.

NanoLC mass spectrometry. Mass spectrometry (MS) was performed with an ion trap mass spectrometer (LCQ Deca XP$^+$; Thermo-electron) equipped with a nanoelectrospray ion source coupled with a nano high pressure liquid chromatography system (LC Packings Dionex). The protein extract was diluted in 50 μL of water, and 1 μL of solution was desalted and concentrated on a C4 reserve phase precolumn (Dionex) with H_2O/acetonitrile-95/5% to 0.1% HCOOH and separated on a C4 nanocolumn Vydac column (Dionex). Proteins were eluted using a 5% to 50% linear gradient of H_2O/Acetonitrile-20/80% to 0.08% HCOOH followed by an isocratic elution with 95% of the same buffer for 10 min. For MS analysis, coated nanoelectrospray needles were obtained from New Objective (Woburn), and the mass spectrometer was operated in positive ionization mode. Data acquisition was performed from a full scan MS over the range m/z 700 to 2,000 units, and a selected ion monitoring (SIM) scan of three ions selected of NGF was monitored. As intact proteins gave multiply charged ions in ion trap, we chose three characteristic multiply charged ions (+8, +9, and +10) of NGF for SIM scan acquisition.

Immunohistochemistry of human breast tumors. Immediately after resection, biopsies were fixed with formalin (10%) for 24 h and then dehydrated through increasing alcohols and embedded into Paraplast Plus. Sections (5 μm) were transferred to Esco Superfrost Plus (Polylabo) slides. For immunohistochemical staining, sections were primarily treated

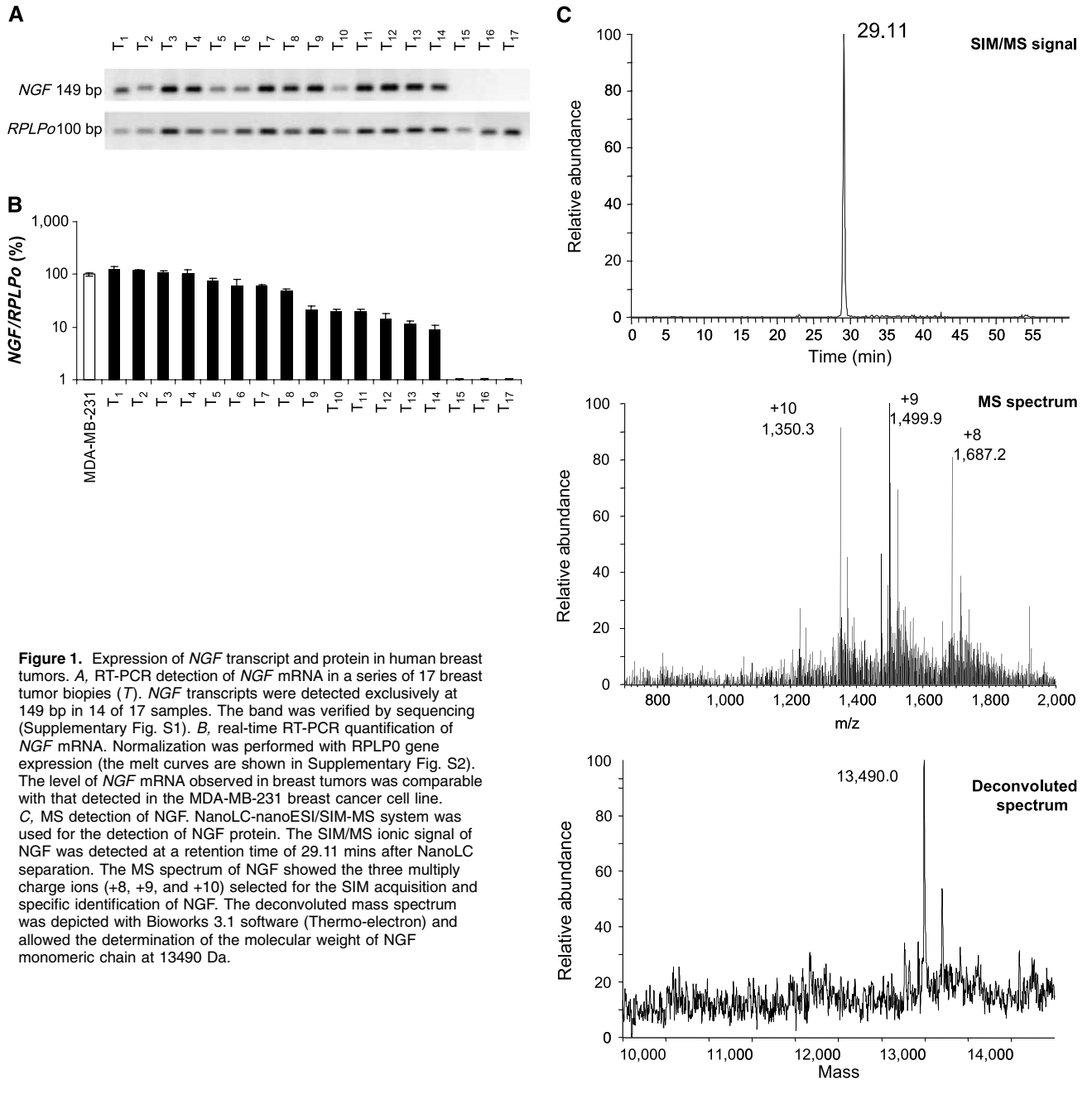


Figure 1. Expression of *NGF* transcript and protein in human breast tumors. *A,* RT-PCR detection of *NGF* mRNA in a series of 17 breast tumor biopies (*T*). *NGF* transcripts were detected exclusively at 149 bp in 14 of 17 samples. The band was verified by sequencing (Supplementary Fig. S1). *B,* real-time RT-PCR quantification of *NGF* mRNA. Normalization was performed with RPLP0 gene expression (the melt curves are shown in Supplementary Fig. S2). The level of *NGF* mRNA observed in breast tumors was comparable with that detected in the MDA-MB-231 breast cancer cell line. *C,* MS detection of NGF. NanoLC-nanoESI/SIM-MS system was used for the detection of NGF protein. The SIM/MS ionic signal of NGF was detected at a retention time of 29.11 mins after NanoLC separation. The MS spectrum of NGF showed the three multiply charge ions (+8, +9, and +10) selected for the SIM acquisition and specific identification of NGF. The deconvoluted mass spectrum was depicted with Bioworks 3.1 software (Thermo-electron) and allowed the determination of the molecular weight of NGF monomeric chain at 13490 Da.

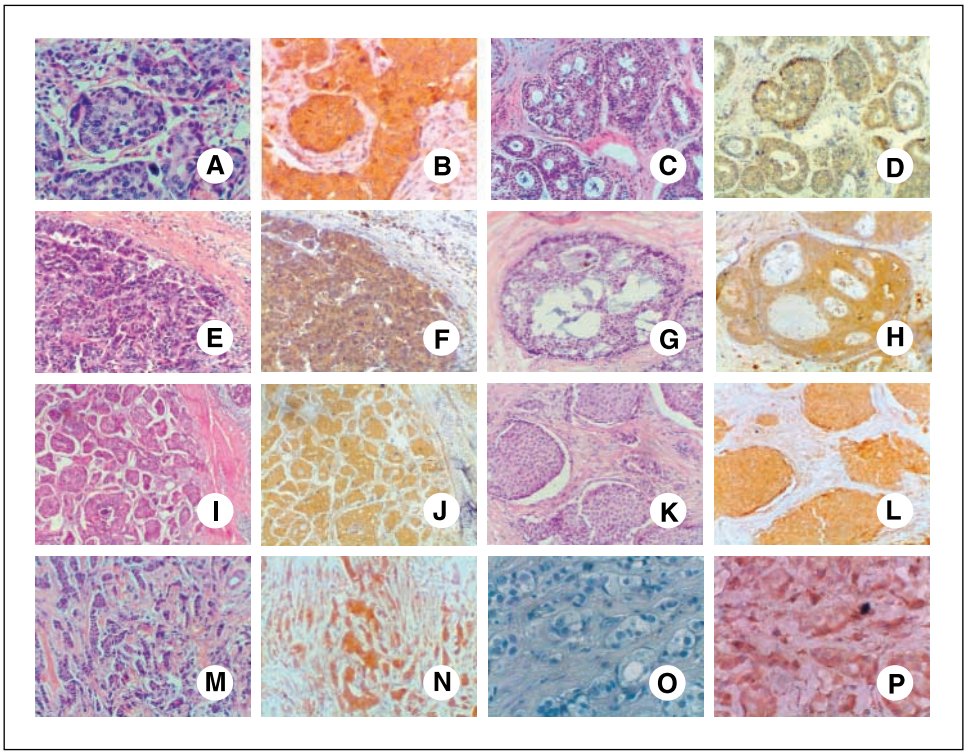


Figure 2. Immunodetection of NGF in human breast cancer tumors. Immunohistochemistry with NGF antibody (*Ab*) was performed on breast cancer biopsies (*a, c, e, g, i, k*, and *m*), and hemalun staining was performed on adjacent sections (*b, d, f, h, j, l*, and *n*). Strong anti-NGF immunoreactivity was observed in all histologic types of breast cancer. *a* and *b,* invasive ductal carcinomas (IDC); *c* and *d,* invasive lobular carcinomas (ILC); *e* and *f,* colloid mucillaneous carcinomas (CMC); *g* and *f,* apocrine carcinomas (AC); *i* and *j,* epidermoid metaplasic carcinomas (EMC); *k* and *l,* tubular carcinomas (TLC); *m* and *n,* intracanalar carcinomas (ICC). The controls of immunohistochemical reaction, without (*o*) and with (*p*) anti-NGF antibodies, are shown in the case of invasive ductal carcinoma. All tissue samples used here have been characterized previously (9).

as described (11) to restore antigen specificity before immunostaining; slides were immersed for 7.5 min in citrate buffer [0.01 mol/L (pH 6)], heated in a microwave, and then placed for 15 min under cold water. Rabbit polyclonal antibodies against NGF (sc-548) were from Santa Cruz Biotechnology. Extravidin peroxidase conjugate and 3, 3′-diaminobenzidine were from Sigma, glycergel was from Dako, and enhanced chemiluminescence reagents were obtained from Amersham Life Science. Immunoreactions were visualized with diaminobenzidine chromogen (Sigma), and sections were poststained with hemalun. Photomicrographs were taken with a phase-contrast microscope connected to an Olympus optical Camedia digital camera.

Effect of anti-NGF treatment on tumor xenograft in immunodeficient mice. MDA-MB-231 human breast cancer cells were obtained from the American Type Culture Collection and routinely grown as previously described (5). Six-week-old female severe combined immunodeficient (SCID) mice were purchased from Charles River Laboratories and acclimatized for at least 2 weeks. Mice were maintained under a 12 h light/dark cycle at a temperature of 20°C to 22°C. Food and water were available *ad libitum*. Mice were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. MDA-MB-231 cells were harvested and resuspended in PBS before s.c. injection into flanks (3×10^6 cells per flank) of 8-week-old SCID mice. Ten days after cell injection, anti-NGF treatments were applied every 3 days. Anti-NGF (12.5 μg; Mab 256 from R&D Systems), or 3 μg anti-NGF siRNA in 100 μL PBS, or 20 nmol/L K-252a were injected as close as possible to the tumor. The injections were repeated every 3 days. The tumor volume was determined every 3 days by measuring the length (l) and width (w) and then calculating the volume as $\pi/6 \times l \times w \times (l+w)/2$. Twelve animals were used in each group and experiments were replicated over three independent trials. Statistical significance between control and transfected cell animal groups was evaluated using Student's t test. Hepatic metastasis rates were macroscopically counted for each animal. For determination of index labeling, anti–Ki-67/MIB-1 (Immunotech) was used for the determination of proliferating cells, and cell apoptosis was measured using terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL). The microvascular density was measured after immunocytochemistry using anti-CD31 (Novus Biological).

Results

Expression of NGF in human breast tumors. *NGF* mRNA was detected in breast tumor biopsies by reverse transcription-PCR (RT-PCR) with a 149-bp band readily elicited (Fig. 1*A*). The sequence of the 149-bp band was then verified (sequencing data are shown in Supplementary Fig. S1). The quantification of expression was obtained after real-time RT-PCR (Fig. 1*B*). The corresponding melt curves, obtained for *NGF* and *RPLP0*, are shown in Supplementary Fig. S2. *NGF* expression was found in 14 of 17 tumors, with levels roughly equivalent to those detected in the MDA-MB-231 breast cancer cell line. The presence of NGF was confirmed at the protein level by mass spectrometric analysis (Fig. 1*C*). NGF protein was detected as a single peak after NanoLC separation as shown in the MS spectrum; the deconvoluted mass spectrum of NGF allowed the precise determination of its molecular weight at 13490 Da, which corresponds to NGF monomeric chains. Together, these data unequivocally show the expression of NGF protein in human breast tumors. However, this type of mass spectrometric analysis cannot be considered as quantitative when applied to complex protein mixtures such as tumoral extracts, and therefore, estimation of NGF level was performed after immunohistochemistry.

Immunohistologic localization of NGF in human breast tumors. To study the distribution of NGF in breast cancers, a series of 77 breast tumor biopsies were analyzed by immunohistochemistry. As shown in Fig. 2, most breast tumors examined exhibited specific NGF immunoreactivity, with labeling mostly localized in the cancer epithelial cells. The level of NGF expression was estimated by microscopic observation, and breast tumors were classified into four different categories depending on the intensity of NGF labeling (Table 1). The results show no relationship between the level of NGF immunoreactivity and tumor size, histologic grade, patient survival, node invasion, menopausal status, estrogen, or

progesterone receptor levels. No correlation was found between between protein and mRNA levels. Thus, whereas NGF overexpression seems to be a general phenomenon in breast tumors, there seems to be no relationship between NGF overexpression and any known prognostic factor.

Effect of NGF targeting on xenograft tumor development. MDA-MB-231 cells were injected into SCID mice, and the effect of treatment with anti-NGF or siRNA against NGF on tumor size and metastasis was monitored. Treatment with anti-NGF or siRNA against NGF resulted in strong and significant decreases in tumor size (Fig. 3*A*) that were depicted at the end of the experiments and after various anti-NGF treatments (Fig. 3*B*). Another xenograft model, T-47D human breast cancer cells, was also tested with similar results (Supplementary Fig. S3). Rates of cell proliferation and cell survival in tumors were then evaluated with anti–Ki-67 immunohistochemistry and TUNEL, respectively (Fig. 3*C*). The results indicated that both anti-NGF treatments induced a decrease in both cell survival and proliferation in breast cancer cells. In addition, microvascular density seemed to be significantly lowered by the anti-NGF treatments, and the number of metastases, as observed in the liver, was also diminished. The control of siRNA efficacy in depleting *NGF* mRNA is shown in Fig. 3*D*. Interestingly, the K252a tyrosine kinase receptor inhibitor, which is a relatively specific blocker of the NGF receptor TrkA at the concentrations used here, also induced a decrease in tumor growth and metastasis, thus reinforcing the idea that NGF stimulation contributes to the growth and dissemination of breast tumors.

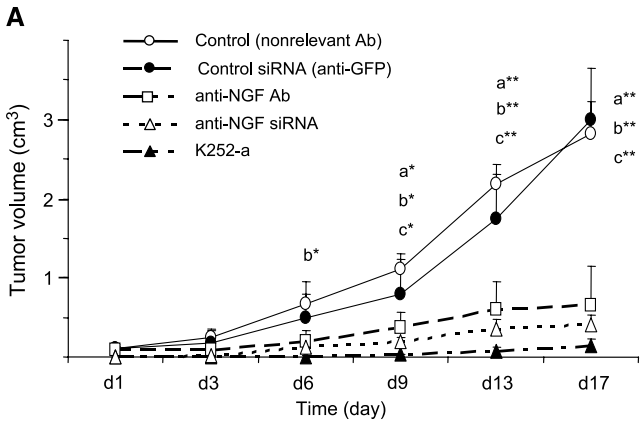
Discussion

As for most growth factors, the biological distribution and activities of NGF have largely superceded the original observation that led to its identification in the first place. NGF was originally discovered as a polypeptide able to stimulate the survival and differentiation of peripheral sympathetic and sensory neurons, but over the last decades, this growth factor has been shown to be expressed in a large variety of nonneuronal cell types and to exhibit other biological activities unrelated to neural system development. Consonant with its wide variety of *in vitro* biological effects, NGF has been shown to be involved in tumorigenesis of both neuronal and nonneuronal cells. Hence, the NGF-TrkA axis is a regulating element for the control of neuroblastoma cell growth, with TrkA alternative splicing able to act as a positive switch for the promotion of neuroblastoma (12, 13). In Wilms tumors, melanoma, medullary thyroid carcinoma, prostatic, and pancreatic tumors, NGF, together with its corresponding receptors, are also expressed

Table 1. NGF overexpression versus various clinicopathologic factors

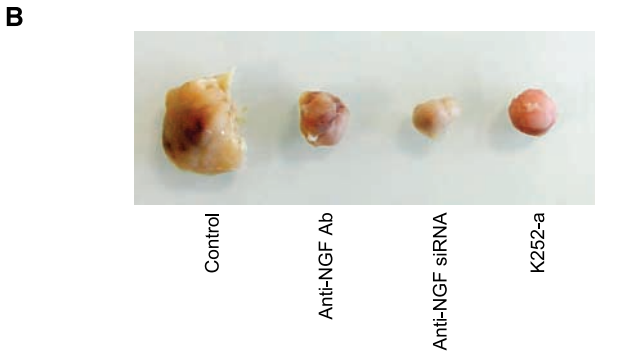
Clinicopathologic factors		NGF labeling intensity				Mean (SD)
		0	1	2	3	
Histologic subclasses of breast tumors	IDC (n = 38)	2	18	7	11	1.7 (0.9)
	ILC (n = 15)	1	10	3	1	1.3 (0.7)
	CMC (n = 5)	0	0	3	2	2.4 (0.5)
	AC (n = 11)	1	0	4	6	2.4 (0.9)
	EMC (n = 2)	0	0	0	2	3.0 (0.0)
	TC (n = 2)	0	2	0	0	1.0 (0.0)
	ICC (n = 4)	1	2	1	0	1.0 (0.8)
Histologic grade (SBR modified)	I (n = 5)	0	2	1	2	2.0 (1.0)
	II (n = 40)	2	16	12	10	1.7 (0.9)
	III (n = 27)	1	12	4	10	1.8 (1.0)
Tumor values	0 (n = 6)	0	4	0	2	1.7 (1.0)
	1 (n = 8)	2	1	1	4	1.9 (1.3)
	2 (n = 43)	2	19	14	8	1.6 (0.8)
	3 (n = 13)	1	5	1	6	1.9 (1.1)
	4 (n = 4)	0	2	0	2	2.0 (1.1)
Survival (5 y)	Alive (n = 64)	4	26	16	18	1.7 (0.9)
	Dead (n = 13)	1	6	2	4	1.7 (1.0)
Lymph nodes status	Positive (n = 35)	1	18	7	9	1.7 (0.9)
	Negative (n = 40)	4	14	9	13	1.8 (1.0)
Menopausal status	Post (n = 50)	4	19	11	16	1.8 (1.0)
	Pre (n = 23)	1	11		6	1.7 (0.9)
Estrogen receptor	Positive (n = 39)	3	16	11	9	1.7 (0.9)
	Negative (n = 36)	2	15	7	12	1.8 (1.0)
Progesterone receptor	Positive (n = 40)	3	15	10	12	1.8 (1.0)
	Negative (n = 35)	2	16	8	9	1.7 (0.9)

NOTE: The intensity of anti-NGF immunohistologic staining in breast tumor biopsies (Fig. 2) was estimated from 0 (no staining) to 3 (intensive staining). For a few patients, some clinicopathologic evaluations failed; consequently, the total number of cases can differ from 77. For each tissue sample, the following clinicopathologic information were obtained: histologic subclassification, tumor (T) values (from the TNM classification of the UICC; ranging from T_0 to T_4), histologic grade (according to Bloom and Richardson), the axillary lymph node status, and the hormone receptor status (estrogen receptor and progesterone receptor).



C

	Control	Anti-NGF antibody	Anti-NGF siRNA	K252-a
% of proliferative cells (Ki-67 detection)	38.8 ± 6	11.95 ± 3	12.7 ± 6	14.6 ± 3
% of apoptotic cells (TUNEL)	2 ± 0.8	12.6 ± 1.3	13.1 ± 2.8	11.4 ± 0.9
Microvascular density (blood vessels/mm^2)	22 ± 4	10 ± 3	3 ± 1.6	12.8 ± 0.6
Number of metastasis (liver)	7 ± 2	2.1 ± 0.5	3.1 ± 1.2	2.3 ± 0.4



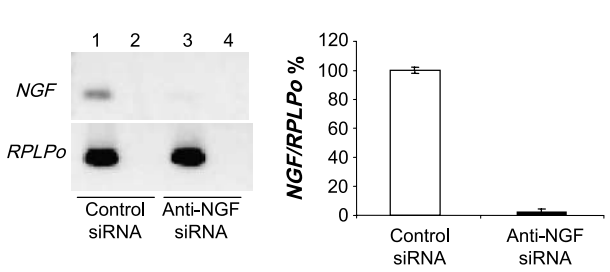


Figure 3. Antitumoral effect of NGF inhibition in animal model. *A,* SCID mice were injected with MDA-MB-231 breast cancer cells. When mean tumor volumes reached about 200 mm^3 in size, mice were injected with 12.5 μg anti-NGF antibodies, 3 μg siRNA against NGF, or 20 mmol/L K252a. Nonrelevant antibody and anti-GFP siRNA were injected as control. All injections were performed peritumorally at days 1, 3, 6, 9, 13, and 17 as indicated. *Points,* mean; *bars,* SD. Twelve animals were used for each group and the experiments were replicated thrice. Student's *t* test was performed between control groups and anti-NGF antibody groups (*a*), between control groups and K252-a groups (*b*), and between anti-*GFP* siRNA groups and anti-NGF siRNA groups (*c*). *, $P < 0.05$; **, $P < 0.01$. The difference between controls and antibody-treated groups as well as siRNA anti-*NGF* groups was significant from day 9 ($P < 0.05$) through days 13 and 17 ($P < 0.01$). Another xenograft model (T-47D human breast cancer cells) was also tested with similar results (Supplementary Fig. S3). *B,* photographic demonstration of comparative tumor size differences observed after anti-NGF treatments (day 17). *C,* evaluation of cell proliferation, apoptosis, microvascular density, and liver metastasis from xenograft tumors. Cell proliferation and apoptosis were measured by immunohistochemistry against Ki67 and TUNEL analysis respectively. Microvascular density was studied after immunohistochemistry for anti-CD31, with liver metastases being macroscopically counted. The results show that anti-NGF antibodies, siRNA against NGF, and K252a treatments all resulted in a decrease of proliferation and an increase in apoptosis of breast cancer cells, accompanied by a diminution in microvascular density and the number of metastases. *D,* demonstration of siRNA efficacy. MDA-MB-231 cells were treated with siRNA against NGF for 48 h, and the quantity of *NGF* mRNA was then assessed by RT-PCR. *RPLPo* was used as control. *Lanes 1* and *2,* control siRNA; *lanes 3* and *4,* siRNA against NGF. *Lanes 1* and *3,* +reverse transcription; *lanes 2* and *4,* -reverse transcription.

(14–19), suggesting the involvement of this growth factor in tumor growth and progression. For breast cancer, it has been shown *in vitro* that NGF promotes both tumor cell survival and proliferation (2–5), and that NGF is overexpressed in breast cancer cells maintained in culture (4). Although NGF receptors have been reported in breast tumors, with a mildly positive relationship to prognosis (6–8), the production of NGF itself had not been described. The ubiquitous expression of NGF that we report here shows that NGF is produced in tumor cells of most breast cancer types. Considering the levels of immunoreactivity that we found in tumor biopsies, the level of expression of this growth factor may well be related to tumor aggressiveness, albeit we have as yet established no relationship with any known prognostic factor. Therefore, although prognostic value is not directly shown here, we have established that NGF expression is directly related to breast cancer.

The detection of NGF in breast tumors further prompted us to explore the hypothesis that it acts to stimulate tumor development. In our animal model, human breast tumor cells were injected, resulting in tumor formation and metastasis. Our data show that inhibiting NGF resulted in a potent inhibition of tumor growth and metastasis. The two approaches that we have used to inhibit NGF activity, blocking antibodies and siRNA, both resulted in a roughly equivalent inhibition of tumor growth and metastasis. In addition, the proportion of proliferating cells in the xenograft tumors was significantly decreased after anti-NGF treatments, with a concomitant increase in the proportion of apoptotic cells, paralleling its stimulating effect on breast cancer cell growth and survival *in vitro* (2–5). Thus, it is possible to posit an NGF autocrine loop that acts to promote survival and stimulate growth of breast cancer cells. This is reinforced by the inhibition of tumor xenografts that we have observed after treatment with the K252a tyrosine kinase inhibitor. Although K252a can inhibit several tyrosine kinase receptors, including Met and the epidermal growth factor receptor, at the concentration used in our studies, its specificity for TrkA is such that it constitutes further support in favor of an NGF autocrine stimulation of breast tumor growth. Interestingly, in some breast tumor specimens, we also observed a diffuse but

specific NGF immunoreactivity in adipocytes (data not shown). The fat tissue surrounding the glandular part of the breast is increasingly being reported as an important regulator of breast epithelial cells, and several lines of evidence suggest its involvement as a regulator of breast cancer cell phenotype and behavior. Further investigation will be necessary to confirm a possible adipocyte-mediated paracrine activity of NGF that involves breast cancer cells. Another interesting feature of our study is the decrease of blood capillary density that we measured in tumor xenografts treated with anti-NGF or siRNA against NGF. NGF has already been posited as angiogenic as it can stimulate proliferation and tubule organization of human umbilical vein endothelial cells (20). In addition, some *in vivo* evidence suggests an NGF-mediated proangiogenic activity in ovarian cancer (21), although it is not yet known if this results from a direct or indirect stimulation of endothelial cells, as NGF can also induce the expression of proangiogenic factors such as vascular endothelial growth factor (11). Nevertheless, the decrease in tumor vascular density that we report here after anti-NGF treatment indicates that perturbing NGF activity provokes not only a decrease in breast cancer cell growth but also a general disturbance of tumor development that can be observed at the level of angiogenesis.

In conclusion, the present work shows the value of NGF as a new molecular target in breast cancer. Although the application of neurotrophin and antineurotrophin strategies have thus far been exploited mainly in the field of neuroscience, our data indicate that interfering with NGF have a direct effect on the development of breast tumors. A variety of approaches have already been developed to modulate the neurotrophic activities of NGF, and developing new contexts for them in breast cancer may open up new opportunities for innovative therapeutic strategies.

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