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Pro-nerve Growth Factor Induces Autocrine Stimulation of Breast Cancer Cell Invasion through Tropomyosin-related Kinase A (TrkA) and Sortilin Protein*[S]

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The precursor of nerve growth factor (proNGF) has been described as a biologically active polypeptide able to induce apoptosis in neuronal cells, via the neurotrophin receptor p75^{NTR} and the sortilin receptor. Herein, it is shown that proNGF is produced and secreted by breast cancer cells, stimulating their invasion. Using Western blotting and mass spectrometry, proNGF was detected in a panel of breast cancer cells as well as in their conditioned media. Immunohistochemical analysis indicated an overproduction of proNGF in breast tumors, when compared with benign and normal breast biopsies, and a relationship to lymph node invasion in ductal carcinomas. Interestingly, siRNA against proNGF induced a decrease of breast cancer cell invasion that was restored by the addition of noncleavable proNGF. The activation of TrkA, Akt, and Src, but not the MAP kinases, was observed. In addition, the proNGF invasive effect was inhibited by the Trk pharmacological inhibitor K252a, a kinase-dead TrkA, and siRNA against TrkA sortilin, neurotensin, whereas siRNA against p75^{NTR} and the MAP kinase inhibitor PD98059 had no impact. These data reveal the existence of an autocrine loop stimulated by proNGF and mediated by TrkA and sortilin, with the activation of Akt and Src, for the stimulation of breast cancer cell invasion.

Nerve growth factor (NGF),[5] the prototypical member of the neurotrophin family of polypeptides, is essential for the survival and differentiation of central and peripheral neurons, and its role in the development and regeneration of the sympathetic and sensory nervous systems has been extensively described (1). NGF binds to the tropomyosin-related kinase A (TrkA) receptor, a tyrosine kinase receptor, and to the p75 neurotrophin receptor (p75^{NTR}), a member of the tumor necrosis factor receptor family, to induce its neurotrophic effects. NGF is synthesized as a 25-kDa precursor protein, named proNGF, that yields the mature NGF polypeptide of 13.5 kDa and an inactive prosegment of 11.5 kDa, released from the N terminus intracellularly by furin, or extracellularly by plasmin as well as by several matrix metalloproteases (2). Importantly, proNGF can be secreted without being processed to mature NGF and can have its own biological effects (3). As more than just a source for NGF, proNGF was shown to induce neuronal death by apoptosis where mature NGF induced survival and differentiation (4, 5). For inducing its proapoptotic effect on neurons, proNGF forms a trimeric complex with two plasma membrane receptors: p75^{NTR} and sortilin (4). Sortilin, a 95-kDa type I receptor, a member of the vacuolar protein sorting-associated protein 10-domain (Vps10p domain) receptors, was first identified for its ability to bind neurotensin and was more recently shown to bind the prosegment of proNGF (4, 6). Interestingly, although p75^{NTR} and sortilin are considered as the main receptors for proNGF, activation of TrkA phosphorylation and downstream signaling has been reported (7, 8). Thus, proNGF, in the absence of processing, is also an active product of the *NGF* gene.

Aside from its neurotrophic properties, NGF has been implicated in a few carcinomas and particularly in breast cancer, where it stimulates both cell proliferation and survival through the activation of TrkA and p75^{NTR}, respectively (9–12). NGF cooperates with the tyrosine kinase receptor HER2 to activate breast cancer cell growth (13), and the anti-estrogen drug tamoxifen, which is widely used in breast cancer therapy, is able to inhibit its mitogenic effect (14). In addition, repression of SHP-1 phosphatase expression by p53 leads to TrkA tyrosine phosphorylation (15). Given TrkA and p75^{NTR} expression in breast tumor cells (16–18), the demonstration that NGF is overexpressed in the majority of human breast tumors and that its inhibition can result in a diminished tumor growth in preclinical models underscores the potential value of NGF as a therapeutic target (19). However, despite these findings with NGF, there has not been any study linking proNGF and breast cancer.

In this study, it is shown for the first time that breast cancer cells release proNGF, producing an autocrine stimulation loop

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[5] The abbreviations used are: NGF, nerve growth factor; pro^{NGF}, precursor of nerve growth factor; TrkA, tropomyosin-related kinase A; NTR, neurotrophin receptor; SIM, selected ion monitoring.

mediated through TrkA plus sortilin and leading to the activation of cancer cell invasion. Thus, these data reveal a direct involvement of proNGF in breast cancer development.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection with siRNA and cDNA Constructs—Breast cancer cell lines were routinely grown as described previously (10). For transfection with siRNA, cells were nucleofected using the Amaxa Cell Line Nucleofector kit V (Lonza) according the manufacturer's recommendations, with 1.5 μg of annealed siRNA. The siRNA sequences used (Eurogentec) were against proNGF (siproNGF) GAAUGCUGAAGUUUAGUCCTT, $p75^{NTR}$ (siP75) AUGCCUCCUUGGCACCUCCTT, and sortilin (siSORT) CUCUGCUGUUAACACCACCTT and compared with control (siGFP) GAUGAACUUCAGGGUCAGCTT. For TrkA, a pool of three siRNA sequences was used: GAACCUGACUGAGCUCUAC, UGGAGUCUCUCUCCUGGAA, and GCUGCAGUGUCAUGGGCAA. The decrease in targeted protein level was assessed by immunoblotting with anti-proNGF (AB9040, Millipore), anti-$p75^{NTR}$ (clone D8A8, Cell Signaling Technology), anti-TrkA (Sc-118, Santa Cruz Biotechnology), and anti-sortilin (612101, BD Biosciences or ANT-009 Alomone Labs, for detection of rat sortilin in PC12 cells). Actin detection (A2066, Sigma-Aldrich) was used for an equi-loading control.

The TrkA expression vector (pDisplay/TrkA) was prepared by inserting TrkA cDNA from MDA-MB-231 cells (TrkA variant 1: NM_001012331.1) into the pDisplay vector (Invitrogen). The kinase-dead TrkA construct was obtained by mutating the three tyrosines 670/674/675 of the tyrosine kinase domain. All other constructs were generated by replacing a single tyrosine residue with phenylalanine with the QuikChange® site-directed mutagenesis kit (Stratagene). Cell transfections were done using Amaxa (Lonza) according to the manufacturer's instructions. Cells were selected with 1 mg/ml G418 (Invitrogen), and the resistant cell populations were stored as frozen stocks and used for all the experiments within 20 passages. Expression of TrkA was not modified with passages as verified by Western blot analysis.

Cell Extracts and Conditioned Medium Preparation—Subconfluent breast cancer cells were rinsed with PBS and lysed in 150 mM NaCl, 50 mM Tris, pH 7.5, 1% SDS, 1% Nonidet P-40, 100 μM sodium orthovanadate and then boiled for 5 min at 100 °C. After centrifugation (12,000 × *g*, 5 min), the supernatant was frozen (−80 °C) until immunoblotting. For conditioned medium recovery, subconfluent cells were rinsed twice and starved for 24 h. The medium was centrifuged (200 × *g*, 10 min, 4 °C), and the supernatant was concentrated by Amicon Ultra-15 with a 10-kDa cut-off according to the manufacturer's recommendations. The concentrated conditioned medium was frozen at −20 °C until immunoblotting or nanoLC mass spectrometry analysis.

Western Blotting—After a 5-min boiling in Laemmli buffer, proteins were submitted to SDS-PAGE and then transferred onto a nitrocellulose membrane by electroblotting. The saturation was done in TBS-Tween 0.1% containing 5% bovine serum albumin, 3% skimmed milk (for anti-TrkA), or 0.2% casein (for anti-sortilin) for 1 h at room temperature. Membranes were incubated with anti-proNGF (AB9040, Millipore), anti-NGF (Sc-548, Santa Cruz Biotechnology) 1/1000, or anti-β-actin antibody (A2066, Sigma-Aldrich) 1/5000 as control, in saturation buffer (overnight, 4 °C). Antibodies against TrkA were from Santa Cruz Biotechnology, and phospho-TrkA (Tyr-674/-675), pan-Akt, phospho-Akt (Ser-473), Src, phospho-Src (Tyr-416), Erk1/2, and phospho-Erk 1/2 (Thr-202/Tyr-204) were from Cell Signaling. The membranes were rinsed and incubated with peroxidase-conjugated anti-rabbit immunoglobulin G antibody (711-035-152, Jackson ImmunoResearch Laboratories) for 1 h at room temperature. After extensive washes, the reaction was revealed using the SuperSignal West Pico chemiluminescent substrate with Amersham Biosciences Hyperfilm ECL or with a Fuji LAS-4000 luminescent analyzer using exposure times at subsaturation.

Mass Spectrometry—A nanoLC-nanoESI/SIM-MS system was used for the detection of proNGF protein, as described previously (19), with an ion trap mass spectrometer (LCQ Deca XP^+; Thermo Fisher) equipped with a nanoelectrospray ion source coupled with a nano high pressure liquid chromatography system (LC Packings, Dionex). The SIM/MS ionic signal of proNGF was detected at a retention time of 29.43 min after nanoLC separation. The deconvoluted mass spectrum was depicted with the Bioworks 3.1 software (Thermo Fisher).

Immunocytochemistry—Cells were grown on rat tail collagen I-coated Labtek dishes until 60% of confluency was reached. After a 1 μM ionomycin (or dimethyl sulfoxide (DMSO) for control) treatment of 60 min, cells were successively rinsed with PBS, pH 7.5, fixed for 20 min with paraformaldehyde 4%, permeabilized for 20 min at room temperature with PBS, pH 7.5; 0.05% saponin; 50 mM ammonium chloride and blocked for 30 min with PBS, pH 7.5; 0.05% saponin; 2% bovine serum albumin. Cells were then incubated for 2 h at room temperature in the blocking solution containing 1/500 proNGF antibody (AB9040, Millipore) or normal rabbit immunoglobulin G (AB-105-C, R&D systems) for control, rinsed with PBS, pH 7.5, and incubated for 1 h at 37 °C in the blocking solution containing 1/2000 Alexa Fluor 488 anti-rabbit immunoglobulin G. Slides were mounted and observed at a 494-nm wavelength through a fluorescence microscope (Nikon).

Immunohistochemistry—Analysis of normal and tumor biopsies was performed using tissue arrays (Biomax and BioChain). After deparaffinization and hydration, antigens were recovered in citrate buffer (10 mM, pH 6) at 60 °C. Endogenous peroxidases were quenched by submerging slides in TBS-Tween 20, 0.1%, containing 3% H_2O_2 (10 min, room temperature). After saturation in TBS-Tween 20 0.1% with 3% BSA (60 min, 37 °C), slides were probed with anti-proNGF antibody (AB9040, Millipore) or with normal rabbit immunoglobulin G (AB-105-C, R&D systems) as control at 1/200 in saturating buffer (2 h, 37 °C). Slides were then washed and incubated with peroxidase-conjugated anti-rabbit immunoglobulin G (711-035-152, Jackson ImmunoResearch Laboratories) at 1/400 in saturating buffer (2 h, 37 °C). Immunostaining was visualized with diaminobenzidine chromogen, and slides were post-stained with Harris hematoxylin. After mounting, photomicrographs were taken.

Cancer Cell Invasion Assay—Cells were rinsed twice and left for 24 h in starvation medium with 0.1% fetal calf serum. Migration assays were done in 12-well Boyden microchambers (Transwell®) with 8-μm pore size membranes. The Transwell microchambers were first coated with 100 μl of starvation medium with 0.1% FCS plus 40 μg of rat tail collagen I for 1 h at 37 °C in 5% CO_2. 100, 000 cells in 400 μl of starvation medium with 0.1% FCS were loaded in the upper chamber, whereas 1.6 ml of starvation medium with 0.1% FCS was placed in the lower chamber. After 20 h of incubation in the presence of proNGF (from Alomone (Israel) and a generous gift from Dr. E. Schwarz, Martin-Luther-University Halle-Wittenberg, Halle, Germany), non-cleavable proNGF (Alomone Labs) including amino acid substitutions or NGF (Scil Proteins GmbH), with or without pharmacological inhibitors (K252a inhibitor of Trk kinase, LY294002 inhibitor of PI3K, PD98059 inhibitor of MAPK, SKI-1 inhibitor of Src, furin inhibitor I, all from Calbiochem) or siRNA sequences, the Transwell microchambers were rinsed with PBS, and the upper surface of the membrane was scraped to remove cells. Neurotensin was from Sigma. The cells remaining on the down side of the membrane were Hoechst-stained and mounted on glass slides with Glycergel before counting (10 fields per membrane) through a fluorescence microscope at 352 nm. Each condition was done in triplicate. Statistics were performed with one-way analysis of variance test and Bonferroni's post test by using GraphPad Prism 5.01 software.

RESULTS

ProNGF Is Produced and Secreted by Breast Cancer Cells—The production of proNGF by breast cancer cells was analyzed by immunoblotting with an anti-proNGF antibody, directed against the prosegment, that does not recognize mature NGF. Each tested cell line was shown to exhibit an immunoreactive band at 25 kDa, which corresponds to the theoretical molecular mass of proNGF (Fig. 1*A*). This band decreased after cell transfection with siRNA targeting proNGF, further demonstrating that it does indeed correspond to proNGF (Fig. 1*B*). In addition, proNGF was not detected in conditioned media when cells were treated with siRNA targeting proNGF. Cellular proNGF localization was assessed by immunocytochemistry (Fig. 1*C*). ProNGF was visualized in the cytoplasm of tested cell lines, whereas normal rabbit immunoglobulin G did not lead to any staining. These data are in accord with our previously published results, which showed that the same cancer cell lines express an *NGF* transcript (10). Moreover, immunocytochemical observations suggested that proNGF was secreted as it was diminished upon treatment with ionomycin, an inducer of secretion. Importantly, Western blot analysis of conditioned medium with anti-proNGF confirmed the presence of an immunoreactive band from four cell lines (Fig. 1*D*). The detection of proNGF in the conditioned media was further validated by anti-NGF recognition of the 25-kDa band. Of note, NGF was also detected at about 14 kDa. Finally, proNGF was detected as a single peak after nano-liquid chromatography separation, as shown in the corresponding mass spectrometric spectra (Fig. 1*E*); the deconvolution of the putative proNGF peak yielded the precise molecular mass (24,928 Da) expected for this protein. Together, these data indicate that proNGF is synthesized and secreted by breast cancer cells.

ProNGF Overexpression in Breast Tumors—The production of proNGF by breast cancer cells was observed in tumor biopsies. Immunohistochemistry was performed on 521 human breast biopsies representing 144 healthy donors, 11 patients with benign tumors, and 366 patients with malignant tumors. As illustrated in Fig. 2*A*, proNGF immunoreactivity was found in the epithelial compartment of various malignant tumors, whereas it was poorly detected in benign tumors and normal biopsies. ProNGF was not found in 94 of 144 healthy donors (65%) nor in 64% of the benign tumor samples. In contrast, proNGF was found in the majority of breast cancers (287 of 366, 78%). Thus, proNGF overproduction appears to be a general phenomenon in a significant number of breast tumors. This is consistent with the reported overexpression of NGF in breast tumors (10, 19). There was no significant correlation between the presence of proNGF and the histological grade, the tumor value, the axillary lymph node status, the presence of metastases, the age, and the presence of estrogen or progesterone receptors (data not shown). Nevertheless, when considering only invasive ductal carcinomas, which represent the majority of breast cancers, a statistically significant association (p = 0.0042) was obtained between the presence of proNGF and lymph node invasion by breast cancer cells (Fig. 2*B*), suggesting a potential link to metastasis.

ProNGF Stimulates Invasion of Breast Cancer Cells through TrkA and Sortilin—The biological impact of proNGF on breast cancer cell invasion was tested. As shown in Fig. 3*A*, non-cleavable proNGF induced a stimulation of breast cancer cell invasion. The data presented in Fig. 3 have been obtained with proNGF from Alomone Labs. In addition, the proNGF 123 construct (20) (generous gift from Dr. Kenneth Neet, Rosalind Franklin University of Medicine and Science, Chicago, IL) was also tested. As shown in supplemental Fig. S1, the results were identical to what was obtained with Alomone Labs proNGF. Non-cleavable proNGF was used here to ensure that no NGF would be generated from proNGF, but it has been observed that wild type proNGF also induced the same stimulation of breast cancer cell invasion; moreover, for the same concentration, NGF did not lead to any significant effect (data not shown). siRNA against proNGF led to a dramatic disruption of cell invasion, which was restored upon the addition of recombinant non-cleavable proNGF, suggesting the existence of an autocrine loop of stimulation. $p75^{NTR}$ impairment by siRNA had little effect, indicating that $p75^{NTR}$ was at best poorly implicated in the proNGF stimulation of breast cancer cell invasion. Interestingly, siRNA against TrkA or sortilin prevented cells from migrating through the collagen-coated membrane of the Transwell microchambers, in the same manner as siRNA against proNGF did in the presence of non-cleavable proNGF. The addition of proNGF was not able to induce cell invasion in the absence of TrkA or sortilin, indicating that these receptors were necessary to trigger the proNGF pro-invasive effect. In contrast to proNGF, when epidermal growth factor was added to sortilin- or TrkA-depleted cells, a stimulation of cell invasion was observed (data not shown). Of note, siRNA against proNGF induced a greater inhibition than that of TrkA, and this could be

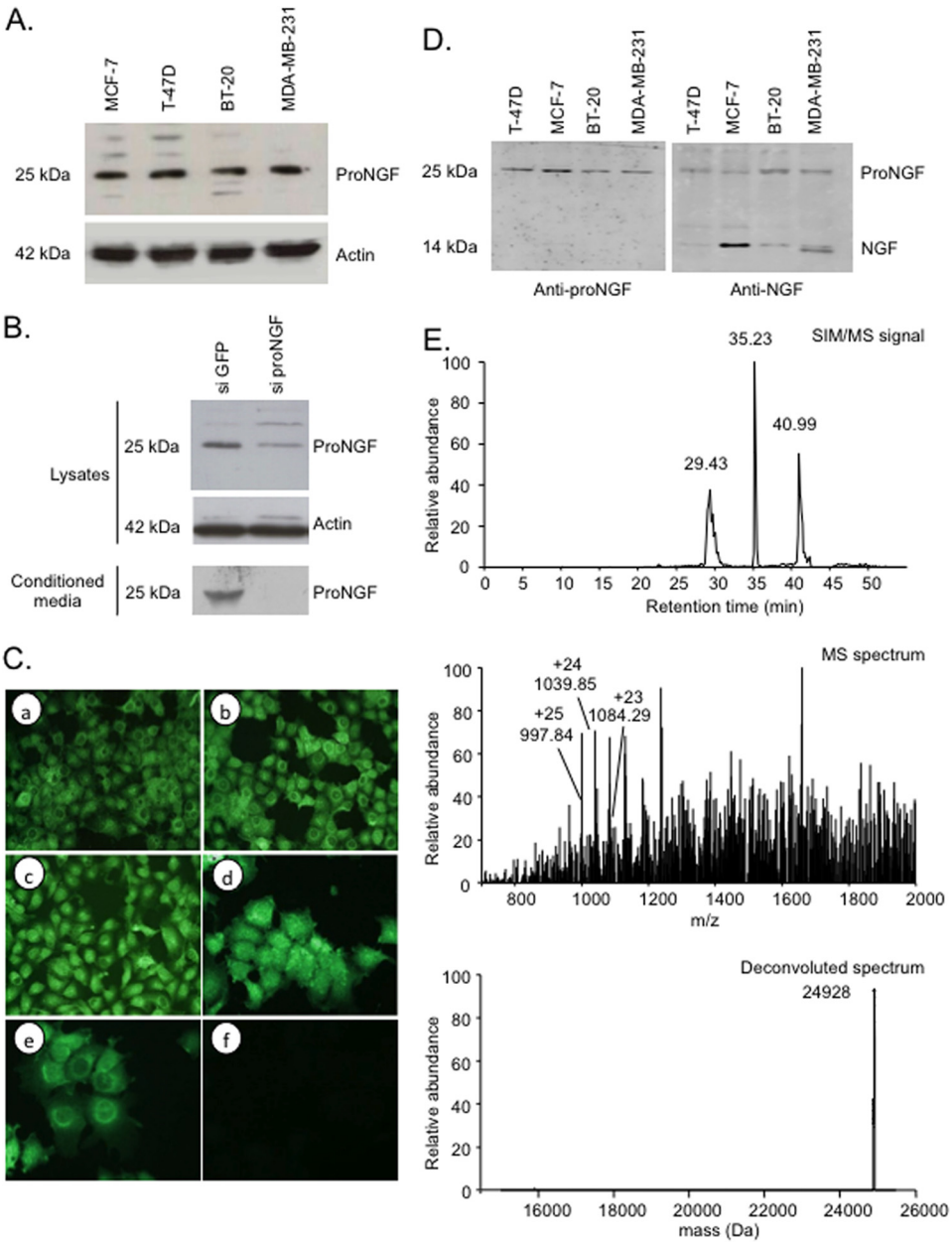
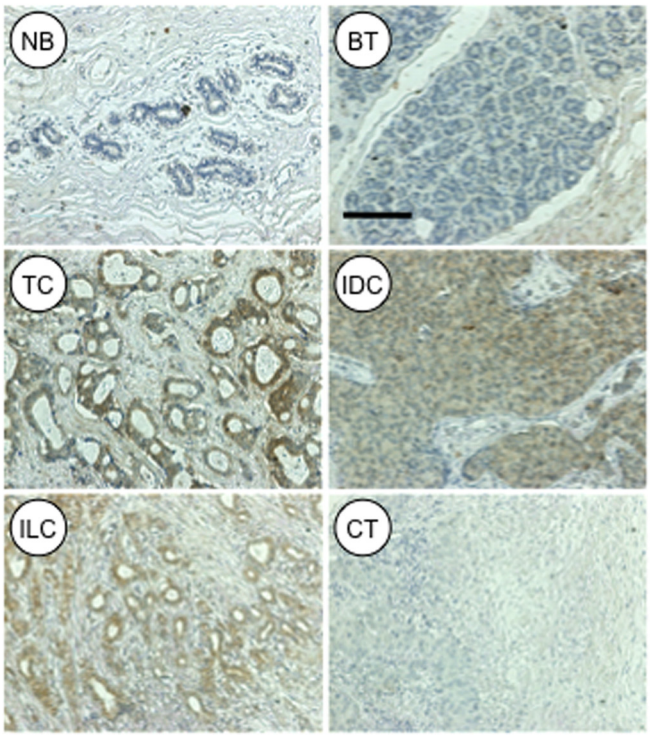


FIGURE 1. **ProNGF production and secretion by breast cancer cells.** *A*, Western blot analysis of proNGF production by breast cancer cell lines MCF-7, T-47D, BT-20, and MDA-MB-231, with 50 μg of protein per lane and anti-proNGF. *B*, Western blot analysis of proNGF in MDA-MB-231 transfected with siRNA against proNGF (*siproNGF*) or control non-targeting siRNA (*siGFP*), in both cell lysate and conditioned media. *C*, *panels a–c*, immunocytochemistry of proNGF in T-47D (*panel a*), BT-20 (*panel b*), and MDA-MB-231 (*panel c*). *Panels d* and *e*, magnification of cellular localization in MCF-7 (*panel d*) and after cells were treated with 1 μM ionomycin (*panel e*). *Panel f*, in control, control MCF-7 cells with rabbit normal immunoglobulin G. *D*, Western blot analysis of conditioned medium of T-47D, MCF-7, BT-20, and MDA-MB-231 cells, with anti-proNGF (*left panel*) and anti-NGF (*right panel*). *E*, mass spectrometric detection of proNGF in breast cancer cell conditioned media. The MS spectrum of proNGF showed the three multiple charge ions (+23, +24, +25) selected for the SIM acquisition and specific identification of proNGF. The deconvoluted mass spectrum shows the molecular mass of proNGF monomeric chain at 24,928 Da.

due to the inhibition of NGF production as NGF can also stimulate breast cancer cell migration (as shown in Fig. 4). In controls, Western blot analysis showed the efficiency of the siRNA in decreasing the level of the different proteins. A Western blot comparison of TrkA, $p75^{NTR}$, and sortilin in breast cancer cells *versus* PC12 cells is presented in supplemental Fig. S2. Together, these results indicate that secreted proNGF is able to stimulate breast cancer cell invasion through TrkA and sortilin rather than $p75^{NTR}$. Significantly, transfection of breast cancer cells with a kinase-dead TrkA abolished the pro-invasive effect of proNGF (Fig. 3*A*), and phosphorylation of TrkA and activation of downstream signaling pathways were thus tested (Fig. 3*B*). ProNGF induced the activation of TrkA, with a maximum level of TrkA phosphorylation observed after 60 min of stimulation that was followed by Akt and Src activation, whereas ERK1/2 were not stimulated. The efficacy and specificity of the pharmacological inhibitors used are shown (Fig. 3*C*), and this is consistent with the fact that pharmacological inhibitors of TrkA, Akt, and Src abolished the proNGF-induced invasion of breast cancer cells (Fig. 3*A*). In contrast, the pharmacological inhibitor of MAP kinases, PD98059, had no impact on proNGF-induced cell invasion. The selective mutation of TrkA Tyr-490/

A.



B.

Clinicopathological factors			proNGF labeling intensity				Mean (SD)
			0	1	2	3	
Histological Grade (SBR modified)	I	(n=20)	0	3	8	9	2.3 (0.7)
	II	(n=117)	11	37	47	22	1.6 (0.8)
	III	(n=18)	5	5	3	5	1.4 (1.2)
Tumor value	1	(n=3)	2	0	0	1	1.0 (1.7)
	2	(n=118)	14	36	41	27	1.8 (1.0)
	3	(n=37)	1	9	14	13	2.0 (0.8)
	4	(n=13)	0	1	4	8	2.6 (0.6)
Age (years)	<50	(n=89)	8	25	29	27	1.8 (0.9)
	>50	(n=85)	9	21	31	24	1.8 (0.9)
Lymph nodes status	Negative	(n=74)	13	22	26	13	1.5 (0.9)
	Positive	(n=98)	4	24	34	36	2.0 (0.8) (**p<0.01)

FIGURE 2. **Immunolocalization of proNGF in human breast biopsies.** *A*, immunohistochemistry with anti-proNGF on normal breast biopsies (*NB*), benign tumors (*BT*), and tubular (TC), invasive ductal (*IDC*) and lobular (*ILC*) carcinomas. As a control (*CT*), normal rabbit immunoglobulin G was used on invasive ductal carcinoma. *Size bar* = 200 μm. *B*, association of proNGF production and clinicopathological parameters in 174 invasive ductal carcinomas. ProNGF labeling intensity was visually determined, from 0 (no staining) to 3 (intense staining), and association with various clinicopathological parameters was tested with the χ^2 test. For some patients, not all data could be retrieved; consequently, the total number can differ. Biopsies are clustered using histological grade (according to Bloom and Richardson), tumor value, patient age, and axillary and lymph node status.

695/751/785 indicated that only Tyr-490 was required for the proinvasive effect of proNGF (Fig. 3*D*). The addition of neurotensin, which is a natural ligand of sortilin, inhibited the phosphorylation of TrkA and the activation of Akt induced by proNGF as well as its proinvasive effect (Fig. 3*E*), suggesting that a proNGF/sortilin interaction is necessary for TrkA activation.

Effect of ProNGF versus NGF on TrkA Signaling and Breast Cancer Cell Invasion—The furin inhibitor I had no impact on either the proinvasive effect of proNGF or the activation of TrkA signaling (Fig. 4*A*). These data indicate that the activation of TrkA signaling by proNGF and the subsequent proinvasive effect do not involve intracellular cleavage into mature NGF. Comparison of proNGF and NGF shows that mature NGF can also induce breast cancer cell invasion, but at much higher concentration (Fig. 4*B*). A maximum effect of proNGF was obtained at 0.5 nM, whereas the maximum effect of NGF was obtained with 16 nM. The proinvasive effect of NGF was inhibited by siRNA against TrkA, but in contrast to what was observed for proNGF, siRNA against sortilin or the addition of neurotensin had no effect on NGF-mediated cell invasion (Fig. 4*C*). The signaling activated by NGF also involves TrkA, Akt, and Src activation (Fig. 4*D*). A side-by-side comparison of the signaling intensity, obtained on the same blot, for proNGF and mature NGF, at concentrations stimulating breast cancer cell invasion (*i.e.* 0.5 nM proNGF and 16 nM mature NGF), showed that the activation of TrkA, Akt, and Src was stronger for mature NGF when compared with proNGF (Fig. 4*E*). Nevertheless, at the concentration for which proNGF is active, mature NGF does not exhibit any activity on either cell invasion or TrkA signaling. Together, these data show that NGF is less potent than proNGF in inducing breast cancer cell invasion and solely involves TrkA activation, without the participation of sortilin.

DISCUSSION

In this study, it is shown that proNGF, which has mainly been studied for its effects in a neuronal context, is produced and secreted by breast cancer cells. Previous studies have reported that cells of the central nervous system can secrete proneurotrophins (21), but to our knowledge, the study reported herein is the first demonstration that proNGF is also secreted by tumor cells. Although the corresponding mature NGF was previously shown to be produced by breast cancer cells (10), the fact that proNGF can be cleaved intracellularly by prohormone convertases and furin could have prevented the secretion of the precursor form (22–24). Nevertheless, proNGF was found in the conditioned medium, and siRNA inhibition resulted in a diminished biological effect, indicating the secretion of proNGF by breast cancer cells.

Although NGF promotes breast cancer cell survival and growth via $p75^{NTR}$ and TrkA, followed by the activation of NF-κB and MAP kinases (9, 11, 25, 26), the results shown here indicate that proNGF activates tumor cell dissemination via TrkA and sortilin, without involvement of $p75^{NTR}$, resulting in Akt and Src activation. $P75^{NTR}$ and sortilin are described as

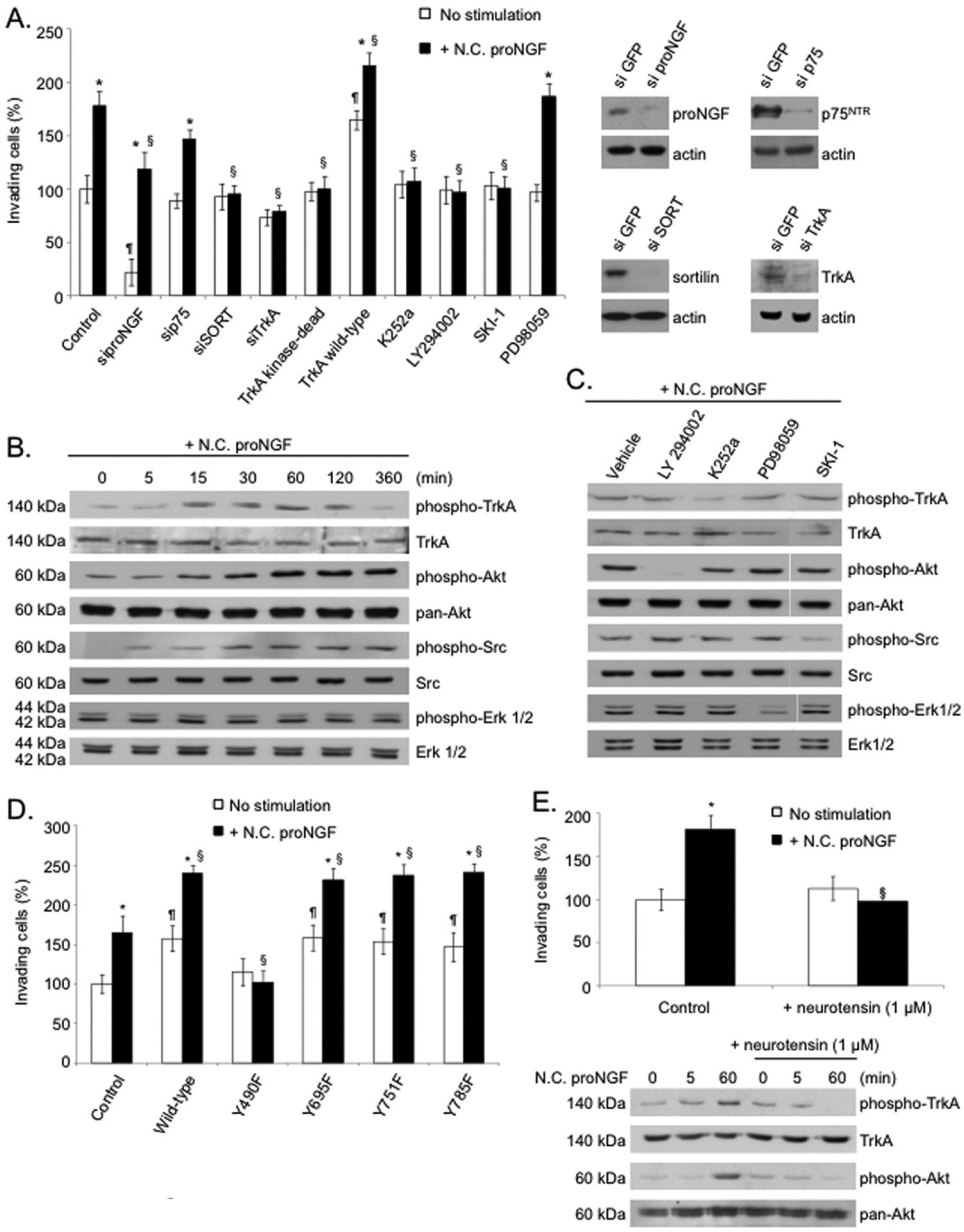


FIGURE 3. **Effect of proNGF on invasion of breast cancer cells and associated signaling pathways.** *A*, cell invasion assay on MDA-MB-231 transfected with the siRNA against proNGF (*siproNGF*), p75^{NTR} (*sip75*), sortilin (*siSORT*), TrkA (*siTrkA*), control siRNA (*Control*), or TrkA kinase-dead *versus* TrkA wild type, treated or not with 0.5 nM recombinant human non-cleavable proNGF (*N.C. proNGF*) and/or 10 nM K252a (Trk inhibitor), 15 μM LY294002 (PI3 kinase inhibitor), 50 nM SKI-1 (Src inhibitor), or 10 μM PD98059 (MAP kinase inhibitor). Untreated siGFP-transfected control cells represent the control 100% of invasion (*white bar*). The efficiency of siRNA treatments was assessed by Western blotting. *B*, proNGF-induced cell signaling in breast cancer cells. MDA-MB-231 cells were stimulated by 0.5 nM non-cleavable proNGF for the indicated times. *C*, the efficacy and specificity of pharmacological inhibitors used in cell invasion assays were tested in Western blotting. *D*, cell invasion assay on MDA-MB-231 cells transfected with mutated forms of TrkA. Tyr-490, Tyr-695, Tyr-751, and Tyr-785 were mutated, and response to proNGF was tested. *E*, neurotensin effect on proNGF-induced signaling and breast cancer cell invasion. For Western blotting and cell invasion assay, the experimental conditions were identical to what was described in *A* and *B*. For the statistics in *A*, *D*, and *E*, *error bars* represent S.D. *, $p < 0.001$ for proNGF stimulation *versus* no stimulation; §, $p < 0.001$ for experimental *versus* control under proNGF stimulation; ¶, $p < 0.001$ for experimental *versus* control with no proNGF stimulation.

receptors of proNGF in the nervous system, and the molecular and structural basis of their interaction has been described (27). In melanoma cells, it has previously been shown that the addition of proNGF can lead to a stimulation of cell invasion through activation of p75^{NTR} and sortilin (28); in this model, TrkA was not found to be necessary. However, the activation of TrkA by proNGF has also been reported in other models (8), with the biological activity of proNGF dependent upon relative levels of TrkA *versus* p75^{NTR} (29). Therefore, although p75^{NTR} appears to be mediating proNGF invasive effects in melanoma, in breast cancer, the present study indicates that TrkA is involved without the participation of p75^{NTR}. Whether or not this is related to a different ratio of TrkA/p75^{NTR} between melanoma and breast cancer cells remains to be elucidated, but a common feature between the two models is the necessity of binding to sortilin. A direct interaction between TrkA and sortilin has recently been reported (30), and such an interaction could also occur in breast cancer cells. The fact that neuroten-

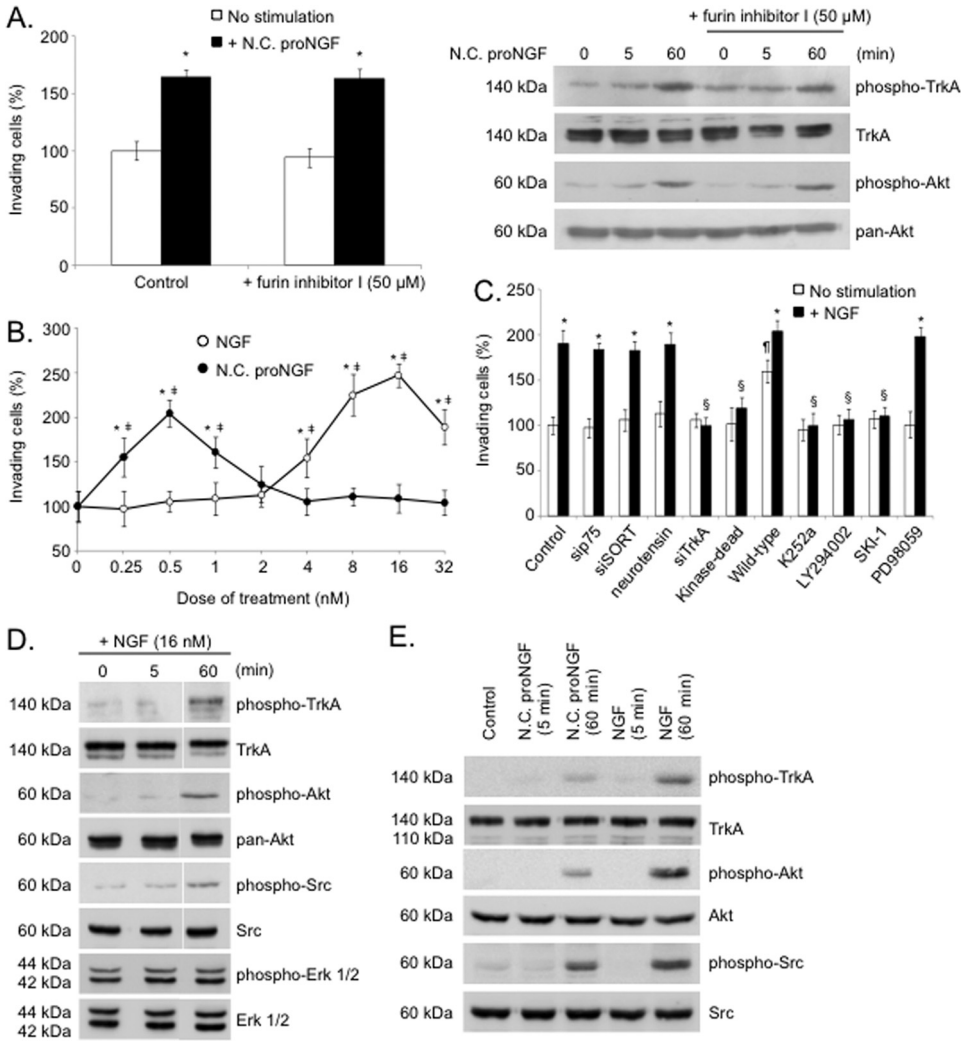


FIGURE 4. **ProNGF *versus* NGF effect on TrkA-mediated signaling and stimulation of breast cancer cell invasion.** *A*, ProNGF does not require cleavage into NGF to stimulate TrkA activation and breast cancer cell invasion. The furin inhibitor I was tested at the indicated concentration on both MDA-MB-231 cell invasion and TrkA signaling activation as described under "Experimental Procedures." *N.C. proNGF*, non-cleavable proNGF. *B*, dose effect of proNGF and NGF on breast cancer cell invasion. The indicated concentrations were tested for 20 h. *C*, cell invasion assay on MDA-MB-231 transfected with the siRNA against $p75^{NTR}$ (*sip75*), sortilin (*siSORT*), TrkA (*siTrkA*), control siRNA (*Control*), or TrkA kinase-dead *versus* TrkA wild type, treated or not with 0.5 nM recombinant human NGF and/or 10 nM K252a (Trk inhibitor), 15 μM LY294002 (PI3 kinase inhibitor), 50 nM SKI-1 (Src inhibitor), or 10 μM PD98059 (MAP kinase inhibitor). Untreated siGFP-transfected cells represent the control 100% of invasion (*white bar*). *D*, NGF-induced cell signaling in breast cancer cells. MDA-MB-231 cells were stimulated with 16 nM NGF for the indicated times. For statistics in *A*, *B*, and *C*, *error bars* represent S.D. *, $p < 0.001$ for proNGF stimulation *versus* no stimulation; $p < 0.001$ for experimental *versus* control under proNGF stimulation; §, $p < 0.001$ for experimental *versus* control under proNGF stimulation; ¶, $p < 0.001$ for experimental *versus* control with no proNGF stimulation. ‡, $p < 0.001$ for proNGF *versus* NGF at the same concentration. *E*, side-by-side comparison of signaling intensity, obtained on the same blot, for proNGF and mature NGF. Concentrations for which a stimulation of breast cancer cell invasion was obtained, *i.e.* 0.5 nM proNGF and 16 nM mature NGF, were used.

sin, the natural ligand of sortilin, was found to inhibit the activation of TrkA by proNGF suggests that a direct interaction of proNGF and sortilin is necessary for the stimulation of TrkA. Moreover, TrkA activation by proNGF has already been observed in PC12 cells, and it requires endocytosis and intracellular proteolysis of the proneurotrophin (7). In breast cancer cells, as demonstrated with the use of non-cleavable proNGFs and the furin inhibitor, the processing of proNGF into NGF is not required for activation of TrkA and cell invasion. Moreover, the activation of breast cancer cell invasion by NGF requires a concentration 15 times higher than proNGF, emphasizing that the generation of mature NGF cannot cause the proinvasive effect of proNGF. Together, this indicates that TrkA is the functional receptor for proNGF in breast cancer cells and that it is involved in the stimulation of tumor cell invasion.

Metastasis of breast cancers is a major issue as it is directly correlated to patient mortality (31). To date, there is no validated biomarker to predict the metastatic nature of breast tumors, and peripheral node invasion by tumor cells remains the criterion used in clinical practice. The observation that proNGF is overproduced in malignant tumors makes it a potentially important molecule in breast cancer development. Importantly, the presence of proNGF was associated with lymph node invasion in invasive ductal carcinomas, whereas the examination of the other breast cancer types did not show such an association. Although further investigation of large cohorts of tumor biopsies will be needed to determine whether proNGF has a practical value as a prognostic biomarker, the data presented here suggest an association between proNGF level and node invasion/metastasis in breast cancer. This

hypothesis is coherent with the *in vitro* effect of proNGF leading to the stimulation of breast cancer cell invasion. In addition, it is also supported by the observation that proNGF induces Src and Akt as these kinases are well known for their involvement in cancer metastases as overexpression or higher activation of both Src and Akt occurs frequently in metastatic cancer cells (32, 33). The activation of Src and Akt downstream to TrkA stimulation has been well described in other models, for instance, in hippocampal neurons (34), and the reported importance of Tyr-490 in activating these pathways is confirmed here. Thus, both *in vitro* and *in vivo* analyses reported in the present study point to a potential role for proNGF in breast cancer metastasis.

In conclusion, proNGF is produced in breast cancer, where it can stimulate tumor cell invasion. ProNGF production adds to the recently reported expression of brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5), showing that neurotrophin family members and their receptors are generally involved in breast cancer (35). Brain-derived neurotrophic factor and NT-4/5 exhibit a different type of activity on breast cancer cells as they were found to stimulate tumor cell survival. In addition, the impact of neurotrophins could go beyond the stimulation of breast tumor cells themselves as recent studies suggest an effect of NGF on angiogenesis (36) as well as on the sprouting of sensory nerve fibers participating in cancer-induced pain (37). In addition to other studies reporting neurotrophin intervention in other pathologies, the present work integrates with the emerging concept that proNGF is a biologically active molecule that can function outside the nervous system. This prompts further studies to determine whether targeting proNGF and its receptors may have clinical benefits in the management of breast cancer, as well as in other carcinomas.

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