

## Brain-Derived Neurotrophic Factor and Neurotrophin-4/5 Are Expressed in Breast Cancer and Can Be Targeted to Inhibit Tumor Cell Survival

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### Abstract

**Purpose:** Given that nerve growth factor has previously been shown to be involved in breast cancer progression, we have tested here the hypothesis that the other neurotrophins (NT) are expressed and have an influence in breast tumor growth.

**Experimental Design:** The expression of brain-derived neurotrophic factor (BDNF), NT-3 and NT-4/5, as well as the neurotrophin receptor p75<sup>NTR</sup>, TrkB, and TrkC, was studied by RT-PCR, Western blotting, and immunohistochemistry in cell lines and tumor biopsies. The biological impacts of neurotrophins, and associated mechanisms, were analyzed in cell cultures and xenografted mice.

**Results:** BDNF and NT-4/5 were expressed and secreted by breast cancer cells, and the use of blocking antibodies suggested an autocrine loop mediating cell resistance to apoptosis. The corresponding tyrosine kinase receptor TrkB was only rarely observed at full length, whereas the expression of TrkB-T1, lacking the kinase domain, as well as p75<sup>NTR</sup>, were detected in all tested breast cancer cell lines and tumor biopsies. In contrast, NT-3 and TrkC were not detected. SiRNA against p75<sup>NTR</sup> and TrkB-T1 abolished the antiapoptotic effect of BDNF and NT-4/5, whereas the pharmacological inhibitors K252a and PD98059 had no effect, suggesting the involvement of p75<sup>NTR</sup> and TrkB-T1, but not kinase activities from Trks and MAPK. In xenografted mice, anti-BDNF, anti-NT-4/5, anti-p75<sup>NTR</sup>, or anti-TrkB-T1 treatments resulted in tumor growth inhibition, characterized by an increase in cell apoptosis, but with no change in proliferation.

**Conclusion:** BDNF and NT-4/5 contribute to breast cancer cell survival and can serve as prospective targets in attempts to inhibit tumor growth. *Clin Cancer Res*; 17(7); 1741–52. ©2011 AACR.

### Introduction

The neurotrophins constitute a family of structurally and functionally related polypeptides including the prototypic nerve growth factor (NGF), as well as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin 4/5 (NT-4/5). They are primarily known for their crucial roles in the development and maintenance of the nervous system, where they stimulate neuronal cell survival, differentiation, and plasticity (1). Neurotrophin activities are mediated through 2 classes of cell surface receptors: the Trk tyrosine kinase receptors and the neurotrophin

receptor p75<sup>NTR</sup>, the latter a member of the Tumor Necrosis Factor-receptor superfamily (2). NGF preferentially binds TrkA, whereas BDNF and NT-4/5 bind TrkB; NT-3 primarily binds TrkC, but also TrkA and TrkB to a lesser extent (3). In contrast, all neurotrophins bind and activate p75<sup>NTR</sup>. Interestingly, non-neuronal cells can also respond to neurotrophins, in both healthy tissues and disease states; in addition to their involvement in neuroblastomas and glioblastomas, several studies have suggested a role for neurotrophins and their receptors in non neuronal cancers (4, 5). Indications that neurotrophins and their receptors can participate in tumorigenesis include data from Wilm's tumors (6), medullary thyroid carcinoma (7), prostatic cancer (8, 9), melanoma (10), myeloma (11), as well as pancreatic (12, 13), ovarian (14), and hepatocellular (15) carcinomas. Nevertheless, these data appear fragmentary, and no comprehensive picture has been established for the involvement of all neurotrophin family members in a defined type of cancer.

In breast cancer, it has previously been shown that NGF is able to stimulate the proliferation and survival of breast tumor cells through the activation of TrkA and p75<sup>NTR</sup>, respectively (16–19). In addition, NGF cooperates with HER2 to activate breast cancer cell growth (20) and the anti-estrogen drug tamoxifen, which is widely used in

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### Translational Relevance

The identification of new molecular markers and targets is crucial for the development of better-targeted treatments for breast cancer. To date, the effort to design such treatments have been limited by the molecular and cellular heterogeneity of breast tumors. The 2 clinically validated molecular targets currently used for this pathology, the estrogen receptors and the tyrosine kinase receptor Erb-B2, have a restricted spectrum of use; thus, the discovery of new targets in breast cancer constitutes a major objective. Here we show that brain-derived neurotrophic factor and neurotrophin-4/5 (NT-4/5) are expressed in breast tumors, promote breast cancer cell survival through p75<sup>NTR</sup> and TrkB-T1, and can be targeted to inhibit tumor growth. Thus, the assaying and targeting of these neurotrophins in breast cancer may have clinical ramifications.

breast cancer therapy, is able to inhibit the mitogenic effect of NGF (21). In addition, repression of SHP-1 phosphatase expression by p53 leads to TrkA tyrosine phosphorylation and the suppression of breast cancer cell proliferation (22). Given the TrkA and p75<sup>NTR</sup> expression in breast tumors (23–25), the demonstration that NGF is overexpressed in the majority of human breast tumors and that its inhibition results in diminished tumor growth in preclinical models, pointed to the potential value of NGF as a therapeutic target (26). With regard to the other neurotrophins, although it has previously been shown that exogenously added BDNF, NT-4/5, or NT-3 can produce antiapoptotic effects on breast cancer cells *in vitro* (16), no studies have systematically investigated the expression of these neurotrophins and their associated receptors in breast cancer cells or their potential subsequent impact on tumor growth.

Here, we report for the first time that BDNF and NT-4/5 are both expressed and secreted by breast cancer cells. An autocrine stimulation loop of BDNF and NT-4/5, mediated through p75<sup>NTR</sup> and TrkB-T1, a variant form of TrkB lacking the kinase domain, was found to be involved in tumor cell survival. These results indicate that BDNF and NT-4/5 directly contribute to breast cancer progression.

### Materials and Methods

#### Materials

Cell culture reagents were purchased from BioWhittaker except fetal bovine serum (Perbio), culture medium (Cambrex), fibronectin (Falcon-Biocoat), insulin, and transferrin (Sigma). The flasks and Petri dishes were obtained from Starstedt (Fisher-Scientific). Recombinant human neurotrophins, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) were from R&D Systems. Ceramide C2 was from Sigma. Antibodies used for immunostaining were rabbit polyclonal anti-BDNF, anti-NT-3, anti-NT-4/5, anti-TrkA, anti-TrkB, and anti-TrkC from Santa Cruz

Biotechnology, p75<sup>NTR</sup> antibody from Promega, anti-actin from Sigma, anti-phosphoTrkA, anti-Erk1/2, and anti-phosphoErk1/2 were from Cell Signaling, and secondary antibodies from Jackson laboratories. ECL reagents were obtained from Pierce Interchim. Neutralizing anti-NT-4/5 and BDNF antibodies were from R&D systems. K252a and PD98059 were obtained from Calbiochem. RNA and histological slides of breast tumor biopsies were from Clin-Sciences. Hoechst 33342, electrophoresis reagents, and chemicals were from Sigma, solvent from Fluka and Glycerol from Dako. DNase and Retro-Transcription reagents were from Invitrogen (Fisher-Scientific) and the Quantitect SYBR Green PCR kit, used for real-time PCR was obtained from Qiagen. Primers for BDNF, NT-3, NT-4/5, p75<sup>NTR</sup>, TrkB, TrkB-T1, TrkB-T-shc, and RPLP0 were from Eurogentec, primers for TrkC from Qiagen. Molecular weight standards were obtained from Fermentas.

#### Cell culture

The human breast epithelial cell lines (MCF-7, T-47D, MDA-MB-231, BT-20, and MCF-10A) and the human neural precursor cells NTERA-2 cl.D1 (NT2/D1) derived from the NT-2 teratocarcinoma were obtained from the American Type Culture Collection. The Human Mammary Epithelial cells (HMEC) were from Cambrex. MCF-7, T-47D, MDA-MB-231, BT-20, and MCF-10A, were routinely grown in monolayer cultures as described previously (17). HMEC and NT2/D1 were grown in conditions given by the supplier. To obtain conditioned media, MCF-7, MDA-MB-231, and HMEC were plated in 175 cm<sup>2</sup> flasks. When they reached confluence, they were washed and incubated in basal medium. Two hours later, the basal medium was changed and cells were further cultured for 24 hours. The medium was then collected, concentrated with column (Amicon), and stored at –80°C before use. To test neurotrophins activities, breast cancer cells were transfected by nucleofection (Amaxa) with siRNA against p75<sup>NTR</sup> 5'-AUGCCUCCUUGGACCUC-3' and 5'-GGAGGUGCCAAGGAGGCAU-3' (siRNA1 p75<sup>NTR</sup>), 5'-AGAGGGCUCG GACAGACA-3', and 5'-UGUGCUGCCGAGCCCUCU-3' (siRNA2 p75<sup>NTR</sup>); 5'-AGCUCCUCUGGGCAGGACC-3' and 5'-GGUCCUGCCCAGAGGAGCU-3' (siRNA3 p75<sup>NTR</sup>); or control siRNA 5'-GCUGACCUGAAGUUCAUC-3' and 5'-GAUGAACUUCAGGGUCAGC-3' (siControl), or TrkB-T1 5'-GGCUGUGGUGCUUGUUGG-3' and 5'-CCAACAAGCACCACAGCCC-3' (siRNA1 TrkB-T1); 5'-UGGGACUGCUGUUGGCUUA-3' and 5'-UAAGCCAACAGCAGUCCCA-3' (siRNA2 TrkB-T1); 5'-GCCUGCAUUAUCUGUGAGC-3' and 5'-GCUCACAGUAUAUGCAGGC-3' (siRNA3 TrkB-T1). Forty-eight hours after transfection, apoptosis of breast cancer cells was induced by TRAIL, which is proapoptotic for breast cancer cells, at 5 ng/mL for 6 hours in serum-free medium. Another proapoptotic agent, the ceramide C2, was also tested at 20 μM for 24 hours. To evaluate the antiapoptotic activity of exogenous neurotrophins, we used the concentration of 200 ng/mL with or without pharmacological inhibitors: 10 nM K252a (inhibitor of Trk receptors) or 20 μM PD98059 (inhibitor of the MAP-Kinases). Antiapoptotic activity of

endogenous neurotrophins was tested using BDNF and NT-4/5 neutralizing antibodies (R&D systems) or nonrelevant antibodies as control, diluted at 1 µg/mL in serum-free medium 45 minutes before inducing apoptosis with TRAIL. For determination of apoptotic cell percentage, all cells (adherent and nonadherent) were fixed with cold methanol (−20°C) during 20 minutes and washed with phosphate-buffered saline (PBS) after staining with 1 µg/mL Hoechst 33342, for 15 minutes at room temperature in the dark. The apoptotic cells, exhibiting condensed and fragmented nuclei, were counted under a Leica fluorescence microscope in randomly selected fields. A minimum of 500–1000 cells was examined for each condition, and results were expressed as a ratio of the total number of counted cells.

### Real-time RT-PCR

Total RNA from cells were isolated with TriReagent (Euro-medex) and RNA from cells and biopsies treated with DNase. RNA from breast tumor tissues corresponded to 1 fibroadenoma, 1 ductal carcinoma *in situ*, 1 adenocarcinoma, 3 invasive ductal carcinomas, 2 invasive lobular carcinomas, 1 mixed ductal and lobular carcinoma, and 1 medullary carcinoma. Reverse transcription was performed with 1 µg of RNAs, 0.5 µg of random hexamers, 200 units of Moloney murine leukemia virus reverse transcriptase for 10 min at 25°C, 50 minutes at 37°C and 15 minutes at 70°C in a final volume of 20 µL. Real-time PCR amplifications were performed using a Quantitect SYBR Green PCR kit with 2 µL of 1/10 cDNA and 500 nM of primers. The primers used were as follows: for *NT-3* transcript 5'-TGGCATCCAAGGTAA-CAACA-3' and 5'-CTCTGTGTGTCGACGAGTTC-3'; *NT-4/5* 5'-AGGCCAAGCAGTCTATGT-3' and 5'-GGTCTCT-CAG-CATCCAGCT-3'; *BDNF* 5'-TGGCTGACACTTTCGAACAC-3' and 5'-CCTCATGGACATGTTGCAG-3'; *p75<sup>NTR</sup>* 5'-ACGGCTACTACCAGGATGAG-3' and 5'-TGGCCTCGT-CGGAATACGTG-3'; *TrkB full length* 5'-AGGGCAACCCG-CCCACGAA-3' and 5'-GGATCGGTCTGGGGAAAAGG-3'; *TrkB-T1* 5'-TAAAACCGTTGGGAACATC-3' and 5'-ACCCATCCAGTGGGATCTTA-3'; *TrkB-T-shc* 5'-ATGAT-GACTCTGCCAGCCCA-3' and 5'-ATCAGGCGGGTCTTGGGGAA-3'; *TrkC* primers were Quantitect Primer Assay: QT00052906 (Qiagen) and for *RPLP0* (human acidic ribosomal phosphoprotein P0), which was used as a reference gene: 5'-GTGATGTGCAGCTGATCAAGACT-3' and 5'-GAT-GACCAGCCCAAAGGAGA-3'. The subsequent PCR conditions were carried out in the following manner: 95°C for 15 seconds, 60°C (or 55°C for Quantitect Primer Assay) for 20 seconds, and 72°C for 30 seconds. Data were analyzed using the MX4000 PCR system software (Stratagene) with the SYBRGreen option (with dissociation curves). Standard curves were performed on serial dilutions of genomic human DNA or RT-transcripts. Values were obtained with the following calculation: ratio = (cycle number – *b/a*) target / (cycle number – *b/a*) reference (where *a* is the slope of the standard curve and *b* is the ordinate of origin). In real-time PCR, Ct (cycle threshold) is defined as the number of cycles required for the accumulation of a fluorescent signal (corresponding to the accumulation of PCR product) to cross the threshold.

Here, the  $\Delta C_t$  corresponding to (Ct interest gene – Ct reference gene) is added for information.

### Western blotting

Protein extraction of subconfluent cells was performed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP-40, 1 mM permethylsulfonate, 1 mM orthovanadate, 1% SDS, 1% protease inhibitors) at 4°C. Insoluble material was removed by centrifugation at 4°C for 15 min at 10,000 × *g* after proteins boiling at 95°C for 5 minutes. Total protein concentration was determined using BCA assay (Sigma). Fifty micrograms of lysates were separated on SDS-polyacrylamide gels (12.5% for NT and 7.5% for receptors), transferred onto a nitrocellulose membrane (0.45µm) (Scheilcher & Shuell) in transfer buffer [48 mM Tris-Base, 39 mM glycine, 0.0375% SDS, 20% (v/v) methanol] and blocked for 2 hours at room temperature in Tris-buffered saline with Tween-20 (TBS-T) (20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween-20) and 5% BSA or skimmed milk. Incubation with primary antibodies was performed in blocking buffer overnight at 4°C. After washing with TBS-T, the membranes were incubated with anti-rabbit IgG peroxidase antibody (Jackson laboratories 1:10,000) for 1 hour at room temperature. The reaction was revealed using the chemiluminescence kit West Pico chemiluminescent substrate (Pierce) and Hyperfilm (Amersham Biosciences).

### Breast tumor immunohistochemistry

Analysis of tumor biopsies was performed using tissue arrays (Superbiochips, Clinisciences), with TSA biotin system kit (PerkinElmer), according to the manufacturer's instructions. Anti-neurotrophins and anti-TrkB rabbit polyclonal antibodies (Santa Cruz) were used at dilution 1/200 in blocking buffer, overnight at 4°C, in a moist chamber. After several washes, slides were incubated for 1 hour with a secondary biotinylated goat anti-rabbit antibody (BD Pharmingen) at a final dilution of 1/200 in blocking buffer at 37°C. After revelation of immunolabeling, sections were counterstained with hematoxylin to contrast cell nuclei and slides were then cover slipped and observed using a Leica light microscope. Photomicrographs were taken with a phase-contrast microscope connected to an Olympus optical Camedia digital camera. Negative controls were obtained by exclusion of the primary antibodies.

### Tumor xenograft growth in immunodeficient mice

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 hours 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 subcutaneous injection into flanks (4 × 10<sup>6</sup> cells per flank) of the animals. Three days after cell injection, anti-NT-4/5 and anti-BDNF treatments

were applied every 3 days. 12.5  $\mu\text{g}$  of antibodies (BDNF: Mab 258; NT-4/5: AF-268-NA from R&D Systems) were injected as close as possible to the tumor. For receptor inhibition, as there is no blocking antibodies against TrkB-T1, we have used a siRNA based approach. One hundred microliters of a buffer at 50  $\mu\text{M}$  of siRNA against p75<sup>NTR</sup> and TrkB-T1 were used for each injection and the experiment was performed in the same experimental conditions as for the inhibition of BDNF and NT-4/5. siRNA control were also injected. The tumor volume was determined every 3 days by measuring the length ( $l$ ) and width ( $w$ ) and then calculating the volume as  $\pi/6 \times l \times w \times (l + w)/2$ . Eight animals were used in each group. For determination of index labeling, anti-PCNA (Pharmingen) was used for the determination of proliferating cells, and cell apoptosis was measured using terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL, Roche).

### Statistics

Data are presented as mean  $\pm$  standard error of the mean (SD). Statistical significance between 2 groups was evaluated using Student's  $t$  test. Asterisks and symbols in figures indicate significant difference between the test group and the control group, according to time points or conditions.

### Results

#### Expression of neurotrophins and their receptors in breast cancer cells

The expression of BDNF, NT-3, NT-4/5, TrkB full length (TrkB-FL), TrkB-T1, TrkB-T-shc, TrkC, and p75<sup>NTR</sup> was examined in the breast cancer cell lines MDA-MB-231, MCF-7, T-47D, BT-20 as well as in the noncancerous breast epithelial cell lines MCF-10A and HMEC, using both real-time RT-PCR and immunoblotting. The results (Fig. 1) indicated that these cell lines expressed both BDNF and NT-4/5 transcripts, visualized at 200 pb and 164 pb, respectively, after RT-PCR (Fig. 1A). All breast epithelial cells, except MCF-10A, expressed the neurotrophin receptor p75<sup>NTR</sup>, whereas TrkB-FL was detected only in MCF-7 cells. TrkC was never observed. In contrast, TrkB-T1 transcripts were observed in all cell lines. TrkB-T-shc was also never detected (data not shown). NT-3, detected at 249 pb, was either low or not expressed, depending on the cell type, and its tyrosine kinase receptor TrkC was not detectable in any of the tested cell lines. Relative mRNA quantifications and  $\Delta\text{Ct}$  (cycle threshold), obtained for each neurotrophin and for p75<sup>NTR</sup>, as well as TrkB-T1 and TrkB-FL, are depicted in Figure 1B. The sequencing of TrkB-T1 product from MCF-7 cells is shown in Supplementary Data S1. Western blotting on total cell lysates revealed 14 kDa bands, corresponding to BDNF and NT-4/5, in all tested cell types (Fig. 1C). In contrast, NT-3 was not detected at the protein level, in line with the low or absent levels of mRNA. Receptor expression profiles were also tested by Western blotting, and both p75<sup>NTR</sup> and TrkB-T1 were detected in all cell types except MCF-10A. Neither TrkB-FL nor TrkC were detected at the

protein level, again confirming data from RT-PCR analysis. In addition, our results show that BDNF and NT-4/5 were detectable in the conditioned media of both normal and breast cancer cells, indicating the active secretion of these neurotrophins (Fig. 1D).

#### BDNF, NT-4/5, and associated receptors in breast tumors

Expression of BDNF, NT-4/5, and associated receptors was detected by real-time RT-PCR in a pilot series of 10 breast tumor biopsies, with a series of 45 breast tumors also being analyzed by immunohistochemistry. The RT-PCR results (Fig. 2A) show that NT-4/5, TrkB-T1, and p75<sup>NTR</sup> transcripts were present in all biopsies studied, whereas TrkB-FL was found only in a few samples. BDNF was detected in 6/10 tumors. We then determined the relative expression levels of BDNF, NT-4/5, p75<sup>NTR</sup>, and TrkB-T1 in these biopsies. The corresponding mRNA levels were very variable, confirming the previously noted molecular heterogeneity among mammary tumors (Fig. 2B). To study the cellular distribution of BDNF and NT-4/5, breast tissue arrays were analyzed by immunohistochemistry. Figure 3 summarizes the results obtained with the different spots of tissue for each tumor. The staining intensities for NT-4/5, BDNF, and TrkB-T1 were concentrated in and around the cancerous cells and were slightly elevated in cancer biopsies compared with normal tissues, whatever the breast cancer type. The distribution/quantification of p75<sup>NTR</sup> was not shown here, as it has been reported previously (24, 25). The levels of NT-4/5 and BDNF were estimated by microscopic observation, and breast tissues were classified into 4 different categories depending on the intensity of labeling. However, the results show no apparent relationship between the levels of BDNF, NT-4/5, and TrkB irrespective of stage, node invasion, estrogen, or progesterone receptors and p53 levels, suggesting no correlation with classical clinicopathological factors (data not shown). Together, these data demonstrate that BDNF, NT-4/5, and TrkB-T1 are expressed in breast tumors.

#### Biological impact of BDNF and NT-4/5 in breast cancer cells

As BDNF and NT-4/5 were found expressed in breast cancer cells, we next tested their effect on cell survival, proliferation, and migration. Our data indicated no effect of exogenously added neurotrophins on breast cancer cell proliferation and migration (data not shown), whereas an antiapoptotic effect was observed, confirming previous data (16). The 2 exogenously added neurotrophins exhibited a rescue effect on TRAIL-treated MCF-7 cells (Fig. 4A). MDA-MB-231 cells and HMEC cells were also tested, as well as the proapoptotic ceramide C2, and the same prosurvival effect of BDNF and NT-4/5 was observed (data not shown). Therefore, the antiapoptotic effect of BDNF and NT-4/5 is not specific of TRAIL, as illustrated with the ceramide C2, which induces apoptosis in breast cancer cells through a completely different mechanism of action. In contrast, no significant antiapoptotic effects of



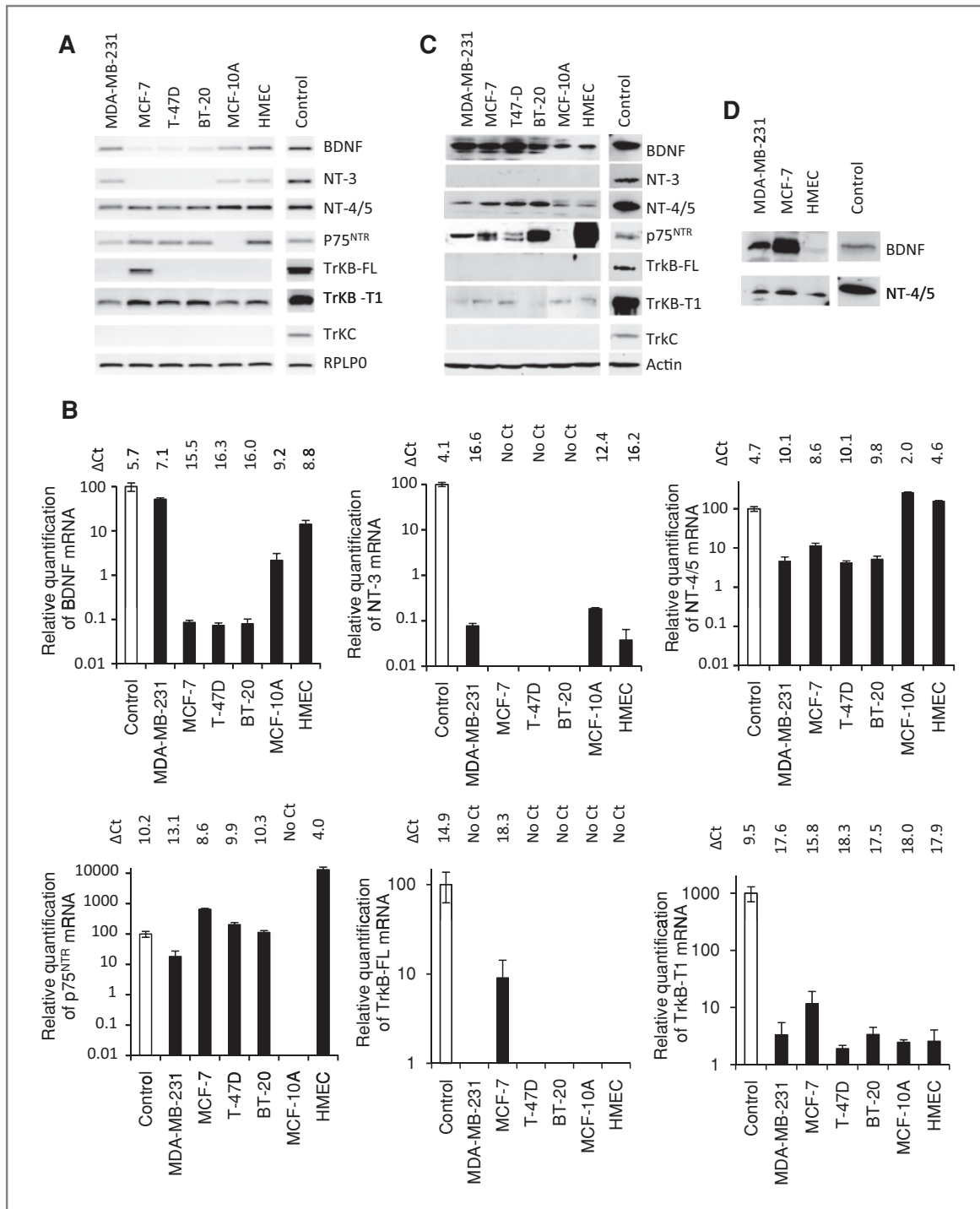


Figure 1. Expression of neurotrophins and their receptors in breast cancer cells *in vitro*. A, total RNA from breast cancer cell lines (MDA-MB-231, MCF-7, T-47D, BT-20) and noncancerous breast epithelial cells (MCF-10A and HMEC), plus the neuronal NT2/D1 cells as control, were isolated and reverse-transcribed. Real-time PCR amplifications of BDNF, NT-3, NT-4/5, p75<sup>NTR</sup>, TrkB-FL, TrkB-T1, TrkB-shc, TrkC, and RPLP0 as loading control, were migrated in 2% agarose gel. B, mRNA relative quantification for BDNF, NT-3, NT-4/5, p75<sup>NTR</sup>, TrkB-FL, and TrkB-T1 in breast cancer cells were calculated as explained in Materials and Methods. The  $\Delta Ct$  are indicated. The control represents value obtained for the NT2/D1 cells. The histogram for TrkC was not presented here because its expression was not found in any cell type. C, protein extracts of various cells were processed for Western blotting using antineurotrophins (anti-BDNF, anti-NT-3, anti-NT4/5) and antireceptors (anti-p75<sup>NTR</sup>, anti-TrkB, anti-TrkC) antibodies. Recombinant proteins or NT2/D1 cells served as positive control and an antiactin antibody was used for equiloading control. D, secretion of BDNF and NT-4/5 by breast epithelial cells. Immunoblotting of conditioned media from MDA-MB-231, MCF-7 cells, and HMEC was performed using anti-BDNF and anti-NT-4/5 antibodies, recombinant BDNF and NT-4/5 proteins were used as control.

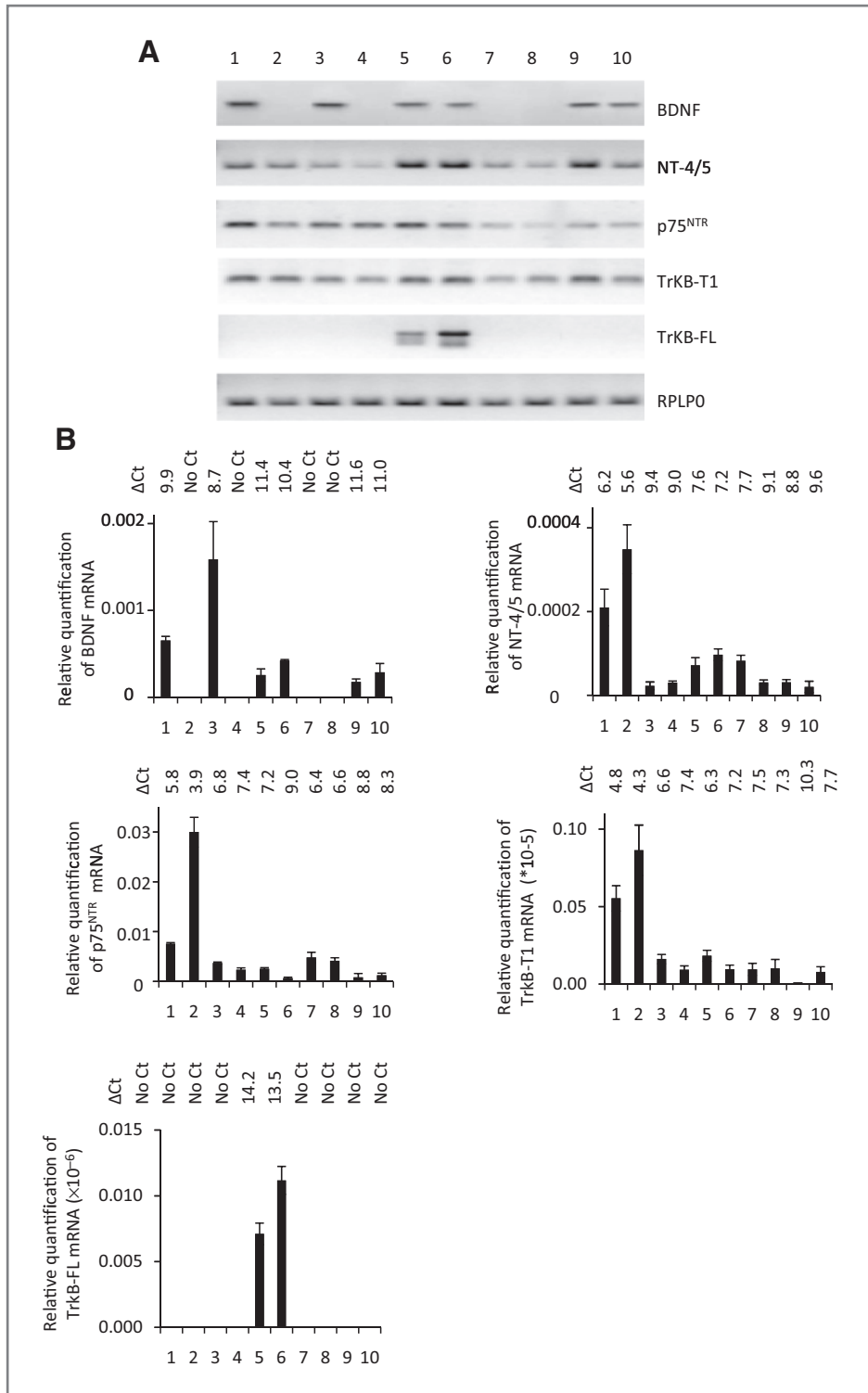
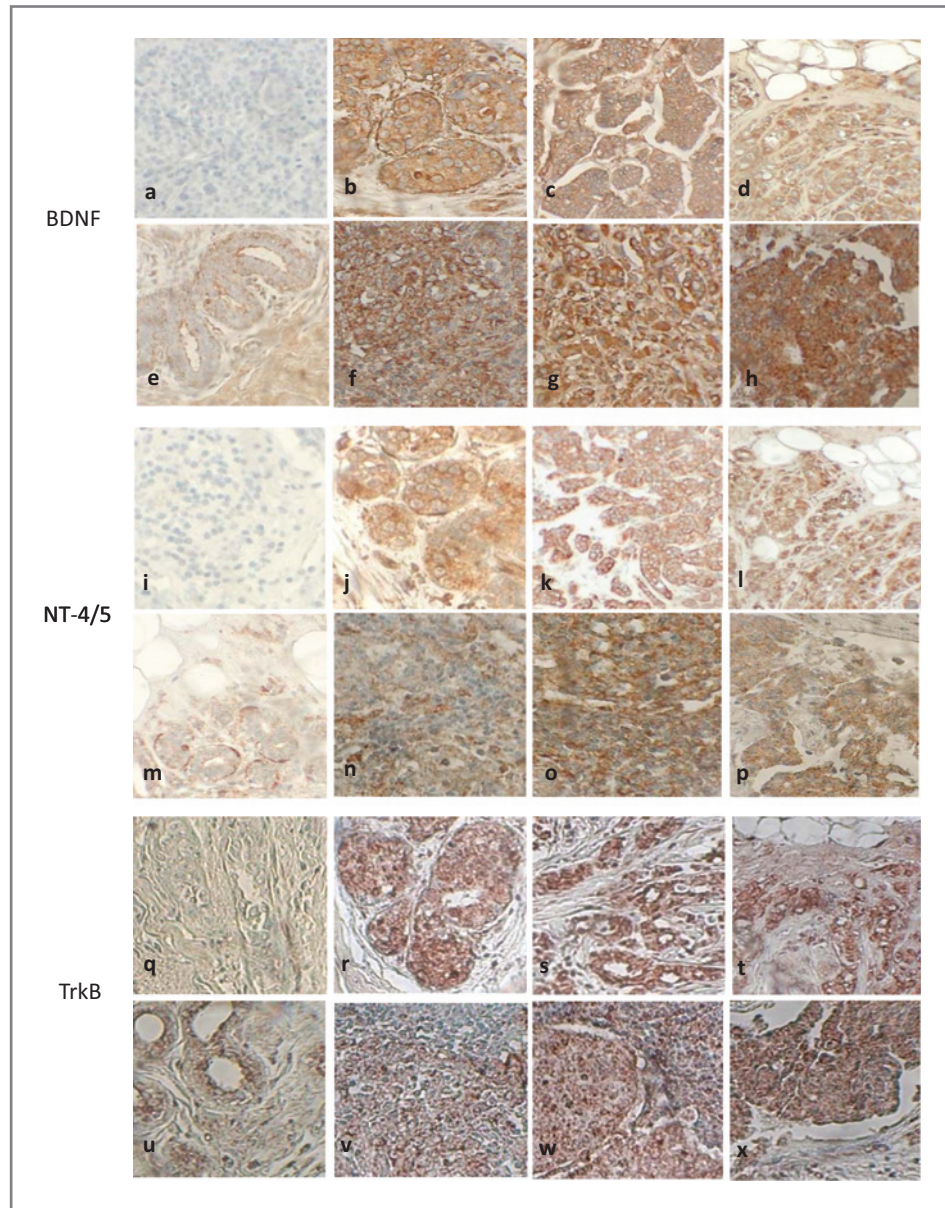


Figure 2. Expression of neurotrophins and their receptors in breast tumors. A, total RNA from 10 breast tumors were reverse-transcribed as described in "Materials and Methods." Real-time PCR amplifications of BDNF, NT-4/5, p75<sup>NTR</sup>, TrkB-FL, TrkB-T1, and RPLP0 as loading control, were migrated in 2% agarose gel. B, relative quantification of neurotrophins and receptors mRNA expression in breast tumors. The ΔCt are indicated.

BDNF and NT-4/5 were observed with MCF-10A cells (data not shown). Interestingly, siRNA against p75<sup>NTR</sup> or TrkB-T1 abolished the NT-4/5 and BDNF antiapoptotic effect (Fig. 4A and B). For each receptor, 3 sequences of siRNA were tested and they all exhibited a similar inhi-

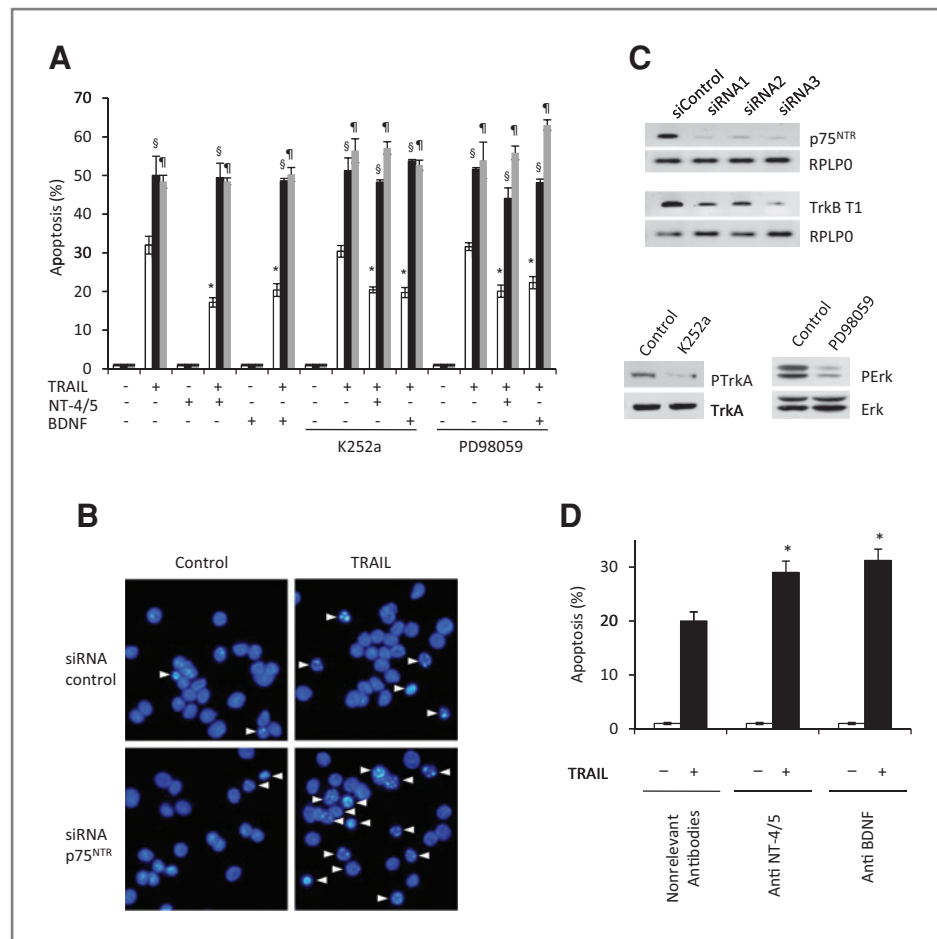
bitory effect. siRNA against p75<sup>NTR</sup> and TrkB-T1 even increased the apoptosis rate compared with siRNA control. Moreover, the Trk pharmacological inhibitors K252a and the MEK inhibitor PD98059 had no impact on the BDNF and NT-4/5 survival effect, indicating the

**Figure 3.** Immunohistochemistry of BDNF, NT-4/5, and TrkB in breast tumors. Antibodies against BDNF, NT-4/5 and TrkB were used on breast tissue arrays. Specific immunoreactivity was observed for all histological types of breast cancer compared to normal breast tissues (e, m, u) or control without primary antibody (a, i, q). b, j, r: ductal carcinoma *in situ*; c, k, s: infiltrating ductal carcinoma; d, l, t: infiltrating lobular carcinoma; f, n, v: metastatic atypical medullary carcinoma in lymph node; g, o, w: metastatic infiltrating ductal carcinoma in lymph node; h, p, x: metastatic infiltrating lobular carcinoma in lymph node.



noninvolvement of either the Trk receptor tyrosine kinase or the MAP kinase pathway. The controls of siRNA and pharmacological inhibitors efficacy are shown in Figure 4C. We then tested the effect of endogenously produced NT-4/5 and BDNF on breast cancer cell growth, through a strategy of inhibition utilizing blocking antibodies. In the absence of exogenously added neurotrophins, the addition of neutralizing anti-NT-4/5 or anti-BDNF resulted in the inhibition of cell survival (Fig. 4D). In the same experimental conditions, antibodies against NT-3 had no effect (data not shown). These data strongly suggest an autocrine loop of BDNF and NT-4/5 resulting in the breast cancer cell survival. This hypothesis was confirmed *in vivo*, with the highly tumorigenic breast cancer cells

MDA-MB-231 which were xenografted in SCID mice. The results indicated that treatments with anti-NT-4/5 or anti-BDNF induced an inhibition of tumor growth, resulting in a decrease of final tumor size (Fig. 5A and B). In addition, the proliferative and apoptotic rates in tumors were assessed by immunohistochemistry with anti-PCNA antibodies and TUNEL staining respectively (Fig. 5C), and the number of brown TUNEL- or PCNA-labeled nuclei counted in each condition. The results, synthesized in Figure 5D, revealed an increase of cell apoptosis in tumor treated with antibodies against neurotrophins, whereas no significant modification of proliferation was observed. In addition, injection of siRNA against p75<sup>NTR</sup> and TrkB-T1 also resulted in an inhibition of



**Figure 4.** Effect of BDNF and NT-4/5 on breast cancer cells *in vitro*. **A**, effect of recombinant BDNF and NT-4/5 on breast cancer cell survival. Thirty-six hours after transfection with siRNA against p75<sup>NTR</sup> (black bars), or TrkB-T1 (gray bars), or control siRNA (white bars), MCF-7 cells were serum-deprived in minimum essential medium overnight and treated with 5 ng/mL TRAIL, with or without 200 ng/mL BDNF or NT-4/5, in presence or absence of 10 nM K252a or 20 μM PD98059 during 6 hours. For p75<sup>NTR</sup> and TrkB-T1, 3 different siRNA sequences, indicated in Materials and Methods, were tested. The results presented here have been obtained with siRNA1 for p75<sup>NTR</sup> and siRNA3 for TrkB-T1. The other sequences exhibited similar effect (data not shown). Apoptotic nuclei were determined after Hoechst staining under a fluorescence microscope. Error bars represent SD and statistics were performed with Student's *t* test. \*, *P* < 0.01 for neurotrophin (BDNF or NT-4/5) stimulation versus no stimulation, in presence of TRAIL; §, *P* < 0.01 for siRNA against p75<sup>NTR</sup> versus control under neurotrophin (BDNF or NT-4/5) stimulation in presence of TRAIL; ¶, *P* < 0.01 for siRNA against TrkB-T1 versus control under neurotrophin (BDNF or NT-4/5) stimulation in presence of TRAIL. **B**, Hoechst staining of MCF-7 cells in control versus siRNA p75<sup>NTR</sup> condition. Apoptotic nuclei, appearing condensed or fragmented, are indicated by arrows. **C**, demonstration of siRNA and pharmacological inhibitors efficacy. MCF-7 cells were treated with siRNA against p75<sup>NTR</sup> or TrkB-T1 and the quantity of p75<sup>NTR</sup> or trkB-T1 mRNA was then assessed by RT-PCR. RPLP0 was used as control. Three different sequences (siRNA1, siRNA2, and siRNA3), indicated in Materials and Methods, were tested for p75<sup>NTR</sup> and TrkB-T1. For pharmacological inhibitors, MCF-7 cells were treated with K252a and PD98059, at concentrations indicated earlier, phospho-Erk1/2 (total Erk1/2 as control) and phosphoTrkA (total TrkA as control) were detected by Western blotting. **D**, effect of blocking antibodies against BDNF and NT-4/5 on survival and resistance to apoptosis of breast cancer cells. As previously described, MCF-7 cells were induced into apoptosis with 5 ng/mL TRAIL, in the absence of exogenously added neurotrophins, with or without neutralizing antibodies against BDNF or NT-4/5, and apoptotic nuclei percentage was determined after Hoechst staining under a fluorescence microscope. \*, *P* < 0.05.

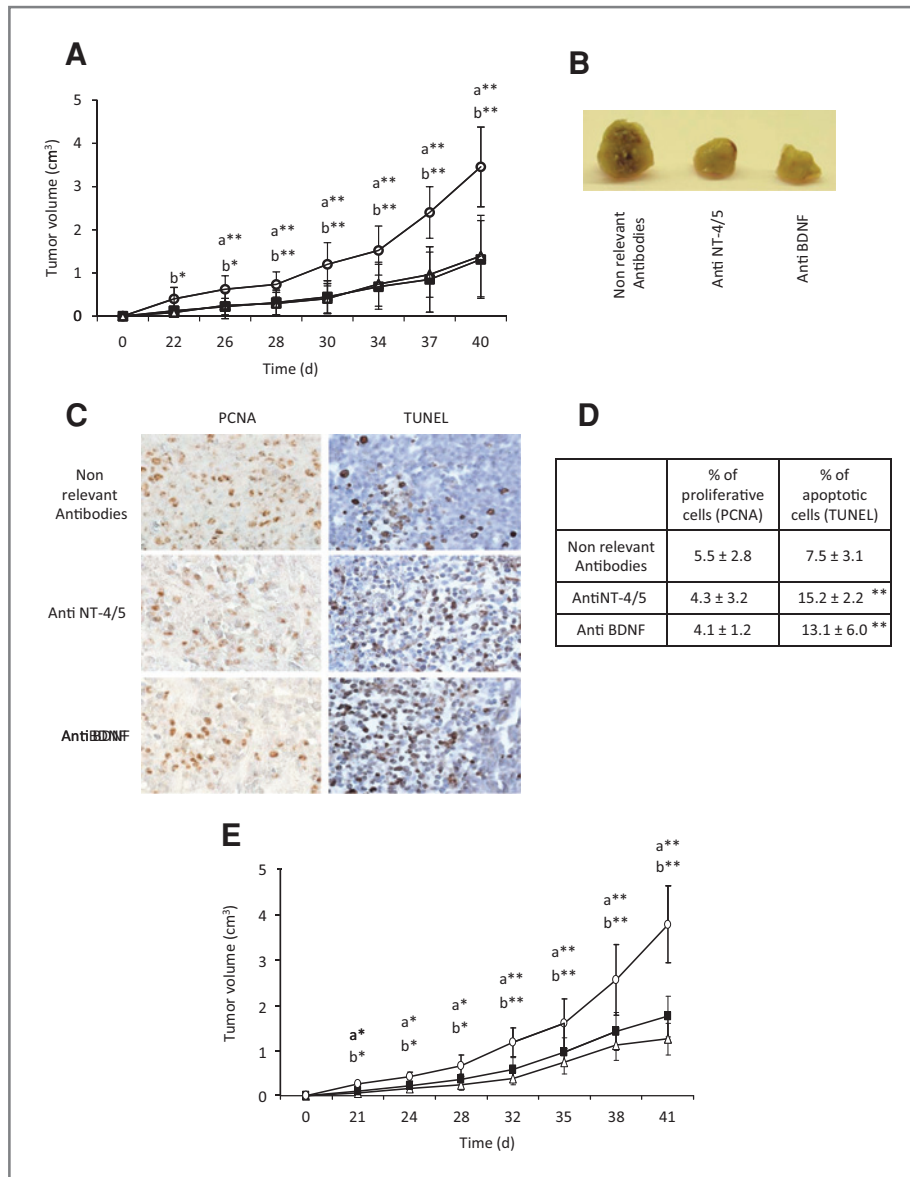
tumor growth (Fig. 5E), confirming the *in vitro* data. Together, these data indicate that BDNF and NT-4/5 stimulate breast tumor cell survival and resistance to apoptosis, through p75<sup>NTR</sup> and TrkB-T1, hence promoting tumor development.

**Discussion**

BDNF and NT-4/5 were first described for their neurotrophic properties, as they contribute to the

development and maintenance of neurons in both central and peripheral nervous systems. They are generally produced by the postsynaptic targets of innervation, allowing nerve fibers to be attracted into and thus for the neuronal cell bodies to survive and establish connections into specific organs and tissues. Our results show for the first time that BDNF and NT-4/5 are also expressed and secreted by cancerous breast epithelial cells, with a widespread expression among both cell lines and tumor biopsies. Unlike NGF, which is overexpressed





**Figure 5.** *In vivo* effect of BDNF and NT-4/5 inhibition on tumor xenograft in immunodeficient mice. **A**, 3 days after subcutaneous injection of MDA-MB-231 cells, SCID mice were injected with 12.5  $\mu$ g of neutralizing antibodies: anti-BDNF ( $\Delta$ ), anti-NT-4/5 ( $\blacksquare$ ), and nonrelevant antibodies ( $\circ$ ) as control. Treatments were repeated every 3 days until animal sacrifice at day 40. Eight animals were used for each group and Student's *t* test was performed between control group and anti-NT-4/5 (a) and anti-BDNF (b) antibodies groups. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . The difference between control and NT-4/5 antibody-treated group was significant from day 26 ( $P < 0.01$ ) through day 40 and between control and BDNF antibody treated group was significant from day 22 ( $P < 0.05$ ) through days 28 to 40 ( $P < 0.01$ ). Experiments were performed twice with equivalent results. The treatment was also effective if started more than 3 days after cell injection. For instance, starting the injection 3 weeks after injection produced the same inhibition of tumor growth. **B**, comparative tumor size differences observed after anti-BDNF or anti-NT-4/5 treatments (day 40). **C** and **D**, cell proliferation and apoptosis were measured by immunohistochemistry against PCNA and TUNEL analysis, respectively. Photographic illustrations of xenografted tumors immunohistochemistry after PCNA and TUNEL analysis are presented in **C**. Evaluations of cell proliferation (PCNA labeling) and apoptosis (TUNEL) from xenograft tumors are in **D**. Three different microscopic fields for each slide, with 3 slides for each tumor, were counted. \*\*,  $P < 0.01$ . **E**, effect of siRNA against p75<sup>NTR</sup> and TrkB-T1 on xenografted MDA-MB-231 cells. The experiment was performed in the same conditions as described in **A**, with injection of 100  $\mu$ L of a buffer at 50  $\mu$ M siRNA against p75<sup>NTR</sup> (siRNA1,  $\Delta$ ), TrkB-T1 (siRNA3,  $\blacksquare$ ), or control siRNA ( $\circ$ ). Eight animals were used for each group and Student's *t* test was performed between control group and siRNA p75<sup>NTR</sup> (a) or siRNA TrkB-T1 (b) groups. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

only in breast cancer cells (17), our results show that NT-4/5 and BDNF are also synthesized and secreted in significant amounts by normal breast epithelial cells, and thus they cannot be pursued as new biomarkers

for breast cancer diagnosis. Considering the levels of immunoreactivity in tumor biopsies, and the strong staining observed in breast metastatic (lymph node) tissue, they may well be related to the degree of tumor

aggressiveness, albeit we have not as yet established any relationship with the known prognostic factors. Therefore, although a direct prognostic value was not shown here, we have nonetheless established that BDNF and NT-4/5 expression occurs in breast tumors.

In contrast to BDNF and NT-4/5, the corresponding tyrosine kinase receptor TrkB-FL was rarely detected, and only at low levels. The involvement of TrkB in neuronal and nonneuronal cancers has been documented, with TrkB being overexpressed in some cancers (neuroblastoma, prostate adenocarcinoma, Wilm's tumors, pancreatic adenocarcinoma, myeloma), resulting in an increased resistance to chemotherapy, and eventually a promotion of tumor invasion, proliferation, and neoangiogenesis (27). However, for breast cancer, this study establishes that there is only a limited expression of TrkB-FL, which was observed in only 1/4 cell lines and 2/10 breast tumors. Expression of TrkB has been reported in mouse and human breast tumors (28, 29), but although these studies did not distinguish the alternative forms of TrkB, they also reported, through Western blotting, the truncated form of TrkB at higher levels of expression than TrkB-FL itself. This prompted us to look for the expression of alternative forms of TrkB. PCR assays were designed to specifically detect TrkB-FL, TrkB-T1, and TrkB-shc. The results showed that TrkB-shc was not expressed, albeit in contrast, we detected TrkB-T1 in all tested breast cancer samples. The other receptor for BDNF and NT-4/5, p75<sup>NTR</sup>, has already been shown to be expressed in breast tumors, and has been shown to have prognostic value (23–25). Supporting this, we confirm here the expression of p75<sup>NTR</sup> in all breast cancer cell lines and tumors.

Several neurotrophic factors and receptors are involved in carcinogenesis (4). In neuroblastoma, the TrkAIII splice variant is able to act as a stimulator, and the BDNF/TrkB axis enhances not only neuroblastoma cell survival, but also resistance to chemotherapy and tumor progression (30–32). In breast cancer, the expression of NGF and glial cell-derived neurotrophic factor (GDNF) increases proliferation, survival, and breast cancer cell scattering (26, 33). Here we show that BDNF and NT-4/5 stimulation could rescue breast cancer cells from apoptosis via p75<sup>NTR</sup> and TrkB-T1, and that they can therefore be considered as prosurvival factors. The pharmacological inhibitors of Trk and MEK had no influence over the BDNF and NT-4/5 prosurvival effect, reinforcing the idea that Trk kinase activities are not involved in BDNF and NT-4/5 signaling in breast cancer cells.

The p75<sup>NTR</sup> receptor regulates neuronal cell apoptosis/survival balance (34) and binds all neurotrophins with the same affinity. In carcinogenesis, p75<sup>NTR</sup> plays complicated roles, as it is on one hand involved in the development of melanoma, through its ability to increase cell invasion, survival, and brain metastasis (35, 10) and yet, on the other hand, it acts as a growth inhibitor in prostate, gastric, and hepatocarcinomas

(36–38). In this study, the inhibition of p75<sup>NTR</sup> or TrkB-T1, with specific siRNAs, abolished the BDNF and NT-4/5 antiapoptotic/prosurvival effect. Abolition of p75<sup>NTR</sup> or TrkB-T1 further increased the basal level of TRAIL-induced apoptosis, suggesting an endogenous stimulation of p75<sup>NTR</sup> and TrkB-T1 through BDNF and NT-4/5 secretion. To date, the biological role of TrkB-T1 has remained elusive. It has been hitherto described exclusively for the nervous system, where its overexpression *in vitro* has been reported to inhibit TrkB-FL and modulate p75<sup>NTR</sup>, leading to neuronal precursor proliferation and differentiation (39–41). Nevertheless, its mechanism of action/intracellular signaling remains unknown. Our data extend the current knowledge about TrkB-T1 by reporting its expression outside the nervous system, it also being the first time that TrkB-T1 involvement is reported for cancer. In addition, we demonstrated that TrkB-T1 is capable of stimulating breast cancer cell resistance to apoptosis, and although its precise mechanism of action has yet to be defined, our data point to a similar effect of inhibiting either TrkB-T1 or p75<sup>NTR</sup>. The hypothesis of BDNF- and NT4/5-mediated autocrine loops was sustained by our *in vivo* experiments, with tumor cell xenograft in immunodeficient mice, wherein blocking anti-BDNF and anti-NT-4/5 antibodies were able to decrease tumor growth. Interestingly, immunohistochemical analysis revealed that anti-BDNF or anti-NT-4/5 treatments induced increase in tumor cell apoptosis, with no effect on the rates of cell proliferation. Therefore, both the *in vitro* and *in vivo* experiments contributed to the evidence for the positive impact of BDNF and NT-4/5 on breast cancer cell apoptotic resistance. Regarding cell signaling involved it was shown, in breast cancer, that p75<sup>NTR</sup> activates NF- $\kappa$ B (18) *via* a mechanism involving BEX2 (19). Interestingly NF- $\kappa$ B and BEX family members are also described for p75<sup>NTR</sup> signaling in neuronal cells (42). In contrast, little is known about TrkB-T1 and in the few studies that have investigated its mechanism of action, a dominant negative function against TrkB has been proposed. Nevertheless, a role of TrkB-T1 in cell survival has already been reported as it has been shown to rescue neuronal cell death by restoring Ca<sup>2+</sup> and restoring BDNF-induced intracellular signaling mediated by full-length TrkB (43). In addition, a very recent study (44) indicates cooperation between p75<sup>NTR</sup> and TrkB-T1 in modulating plasticity in hippocampal neurons. In our study, we found that the inhibition of p75<sup>NTR</sup> or TrkB-T1 both resulted in a complete inhibition of BDNF and NT-4/5 antiapoptotic effect, suggesting a cooperation between p75<sup>NTR</sup> and TrkB-T1 or a cross-linking of their signaling systems; however, further mechanistic investigations will be necessary to solve this question.

In conclusion, our study reveals that BDNF and NT-4/5 are involved in breast cancer. The stimulation of a resistance to apoptosis by BDNF and NT-4/5, through p75<sup>NTR</sup> and TrkB-T1, suggests their potential value as therapeutic

targets in breast cancer that offer new directions for the design of innovative strategies based on neurotrophin inhibition.

### Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

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