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# Nerve growth factor (NGF)-mediated regulation of p75$^{NTR}$ expression contributes to chemotherapeutic resistance in triple negative breast cancer cells

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ABSTRACT

Triple negative breast cancer [TNBC] cells are reported to secrete the neurotrophin nerve growth factor [NGF] and express its receptors, p75 neurotrophin receptor [p75$^{NTR}$] and TrkA, leading to NGF-activated pro-survival autocrine signaling. This provides a rationale for NGF as a potential therapeutic target for TNBC. Here we show that exposure of TNBC cells to NGF leads to increased levels of p75$^{NTR}$, which was diminished by NGF-neutralizing antibody or NGF inhibitors [Ro 08-2750 and Y1086]. NGF-mediated increase in p75$^{NTR}$ levels were partly due to increased transcription and partly due to inhibition of proteolytic processing of p75$^{NTR}$. In contrast, proNGF caused a decrease in p75$^{NTR}$ levels. Functionally, NGF-induced increase in p75$^{NTR}$ caused a decrease in the sensitivity of TNBC cells to apoptosis induction. In contrast, knock-down of p75$^{NTR}$ using shRNA or small molecule inhibition of NGF-p75$^{NTR}$ interaction [using Ro 08-2750] sensitized TNBC cells to drug-induced apoptosis. In patient samples, the expression of *NGF* and *NGFR* [the p75$^{NTR}$ gene] mRNA are positively correlated in several subtypes of breast cancer, including basal-like breast cancer. Together these data suggest a positive feedback loop through which NGF-mediated upregulation of p75$^{NTR}$ can contribute to the chemo-resistance of TNBC cells.



## 1. Introduction

Breast cancer [BC] is the most common cause of cancer death in women [1] and is often classified based on the expression of key receptors, estrogen receptor [ER], progesterone receptor [PR] and human epidermal growth factor receptor-2 [HER2] [2,3]. Triple negative breast cancers [TNBCs], which lack expression of ER, PR or HER2 overexpression [2], are the most difficult to treat because of the lack of targeted therapies [4].

Neurotrophins are a small family of polypeptides, well characterized in their abilities to regulate the survival, development and functions of neurons [5,6]. The neurotrophin family comprises nerve growth factor [NGF], brain derived-neurotrophic factor [BDNF], neurotrophin-3 [NT-3] and neurotrophin 4/5 [NT-4/5] [7]. They can bind to two distinct classes of receptor, the p75 neurotrophin receptor [p75$^{NTR}$] which can bind all neurotrophins with approximately equal affinity [8], and the tropomyosin related kinase [Trk] receptors which exhibit ligand specificity, with TrkA binding NGF, TrkB binding BDNF and NT4/5, and NT3 binding TrkC [9].

NGF is produced by over 80% of primary breast tumors, giving it a potentially broader target range than ER or HER-2 [10]. Pro-survival NGF signaling mediated by p75$^{NTR}$ may contribute to the resistance of breast tumors to chemotherapy [10]. TNBC cells are reported to secrete NGF and express its receptors, p75$^{NTR}$ and TrkA, leading to NGF-activated autocrine signaling [11]. In contrast, normal breast cells do not secrete NGF, although they express both TrkA and p75$^{NTR}$ receptors [12]. Thus, anti-NGF therapy holds the possibility of increasing the effectiveness of cytotoxic/genotoxic drugs used as adjuvant therapies in BC treatment [10].

Transcriptional upregulation of p75$^{NTR}$ expression has been shown to occur in neurons following brain acute injury [13–15]. In addition, p75$^{NTR}$ is also a substrate of receptor intramembrane proteolysis (RIP), involving ectodomain shedding and release of the intracellular domain [16]. In prostate cancer, where loss of p75$^{NTR}$ expression is correlated with metastatic disease, exogenous NGF can upregulate p75$^{NTR}$ expression and reduce cell malignancy [17]. The aim of the current work is to investigate whether NGF is involved in the regulation of p75$^{NTR}$ levels in BC cells. We show that

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NGF can increase the levels of full length $p75^{NTR}$ [FL-$p75^{NTR}$] in BC cells and that this is linked to increased resistance of TNBC cells to chemotherapeutic drug-induced cell death.

## 2. Experimental procedures

### 2.1. Materials

All chemicals were obtained from Sigma-Aldrich unless otherwise indicated. Human breast adenocarcinoma cell lines, MDA-MB-231 and MCF-7 were from European Collection of Cell Cultures and HCC1806 were from American Type Culture Collection. Small molecule inhibitors were as follows: Epoxomicin, GM6001 and DAPT [Calbiochem], Ro 08-2750 [Tocris Biosciences], Y1036 [Merck Millipore]. Mouse NGF 2.5S, human non-cleavable proNGF and NGF/proNGF neutralizing antibody were purchased from Alomone Labs. Rabbit polyclonal antibodies against $p75^{NTR}$ and Actin were from Merck-Millipore and Sigma-Aldrich respectively. All secondary antibodies conjugated to horseradish peroxidase were from Jackson ImmunoResearch Laboratories. Anti-NGF [capture antibody for ELISA] was from Santa Cruz Biotechnology Ltd., hβNGF [used for generating standard curve] was from Alomone labs, biotin-tagged anti-NGF antibody [detection antibody] and Strep-HRP were obtained from R&D Systems. Turbofect transfection reagent was from Fermentas. shRNA $p75^{NTR}$ was from GeneCopoeia. RT-PCR primers were from Sigma-Aldrich. 3, 3′, 5, 5′- Tetramethylbenzidine [TMB] was from Thermoscientific.

### 2.2. Cell culture and treatments

MDA-MB-231 cells and MCF-7 cells were cultured in Dulbecco's Modified Eagle medium [DMEM] and HCC1806 were cultured in RPMI-1640, both supplemented with 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$. Cells were seeded at $3 \times 10^4$ cells/$cm^2$.

### 2.3. Flow cytometry-based assays

MDA-MB-231 cells were treated as described and harvested by trypsinization. For TMRE assay, cells were incubated with 100 nM TMRE for 30 min in the dark. For Sub G1 analysis, cells were centrifuged at 300*g* for 5 min and the pellet fixed in 70% ice-cold ethanol and left at −20 °C. On the day of analysis, cells were centrifuged at 5000*g* for 5 min and re-suspended in 50 μg/ml propidium iodide [PI] stain and incubated in the dark for 30 min. Fluorescence of the cells was measured at 582 nm by flow cytometry. Analysis was carried out using Cyflogic 1.2.1 software and statistical analysis was carried out using GraphPad Prism.

### 2.4. Immunoblotting

Following experimental treatments, cells were lysed in whole cell lysis buffer (20 mM HEPES pH 7.5, 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM ethylene glycol tetraacetic acid (EGTA), 1% Nonidet P-40, 0.5 mM DTT, 0.1% phenylmethylsulphonyl fluoride (PMSF), 1% aprotinin, 5 mM NaF, and 1 mM $Na_3VO_4$). Protein quantification was performed using Bradford reagent with bovine serum albumin as the standard. Proteins (30 μg) were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes. Membranes were probed with antibodies against $p75^{NTR}$ (1:1000) (Millipore, 07-476) and actin (1:1000) (Sigma, A2066) overnight at 4 °C followed by appropriate horse radish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch laboratories Inc.). Protein bands were visualized using Western Lightning ECL substrates (Perkin Elmer, NEL102001EA).

### 2.5. RNA extraction and RT-PCR

Total RNA was isolated using Trizol. Reverse transcription was carried out with 2 μg total RNA and oligo [dT] using 20 U Superscript III Reverse Transcriptase [Invitrogen]. cDNAs were amplified during 32 cycles with the following primers: NGF sense-primer [5′-ATACAGGCGGAACCACACTC-3′]; NGF anti-sense primer [5′-TGCTCCTGTGAGTCCTGTTG-3′]; BDNF sense-primer [5′-TACTTTGGTTGCATGAAGGCTGCC-3′]; BDNF anti-sense primer [5′-ACTTGACTACTGAGCATCACCCTG-3′]; $p75^{NTR}$ sense-primer [5′-GTGGGACAGAGTCTGGGTGT-3′]; $p75^{NTR}$ anti-sense primer [5′-AAGGAGGGGAGGTGATAGGA-3′]; GAPDH sense-primer [5′-ACCACAGTCCATGCCATC-3′]: and GAPDH anti-sense primer [5′-TCCACCACCCTGTTGCTG-3′].

### 2.6. Transient transfection of MDA-MB-231 cells

MDA-MB-231 cells were transfected with 1 μg of shRNA $p75^{NTR}$ or scrambled shRNA that expresses eGFP (sc.eGFP) reporter gene using Turbofect transfection reagent and a DNA to lipid ratio of 1:2. The media was removed 5 h after transfection. Typical transfection efficiency obtained was ~70%.

### 2.7. Determination of NGF concentration

Media from TNBC cells was collected and NGF/proNGF levels in culture media were quantified using an enzyme linked immunosorbent assay [ELISA] kit [R&D System] according to manufacturer's instructions.

### 2.8. Determination of caspase-3 activity using DEVDase assay

HCC1806 cells were treated as described and harvested by trypsinization. The activity of caspase-3 like enzymes [DEVDase activity] was determined fluorometrically as reported previously [18].

### 2.9. Nuclear morphology assay

Treated cells were harvested by trypsinization and fixed in 3.7% paraformaldehyde for 20 min. DAPI in VECTASHIELD, 3 μl, [Vector Laboratories Ltd] was used to mount the slides. DAPI-stained nuclei were visualized using an Olympus IX51 fluorescence microscope [excitation 360 nm, emission 460 nm].

### 2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Values are expressed as means ± SEM of ≥3 separate experiments. Data were analyzed using One-Way ANOVA followed by Tukey's multiple comparisons *post hoc* test unless otherwise stated. Differences were considered statistically significant at $p < 0.05$.

## 3. Results

### 3.1. Neurotrophins mediate increased expression of $p75^{NTR}$ during in vitro culture of TNBC cells

We examined the expression of $p75^{NTR}$ in a panel of BC cells that included MCF-7 [Luminal A], MDA-MB-231 [TNBC] and HCC1806 [TNBC]. During 3 days in culture, the expression of $p75^{NTR}$ increased