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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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Running title: NGF regulates $p75^{NTR}$ expression

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.

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 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.

Experimental Procedures:

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.

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 ºC in a humidified atmosphere of 5% CO_2. Cells were seeded at $3x10^4$ cells/cm^2.

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 X g for 5 min and the pellet fixed in 70% ice-cold ethanol and left at -20 ºC. On the day of analysis, cells were centrifuged at 5000 X 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.

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 ^{o}C followed by appropriate horse radish peroxidase-conjugated secondary antibody (Jackson). Protein bands were visualized using Western Lightning ECL substrates (Perkin Elmer, NEL102001EA).

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'].

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%.

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.

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).

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].

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Values are expressed as means ± SEM of $\geq$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 $p<0.05$.

Results

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 in both of the TNBC cell lines, while in contrast, $p75^{NTR}$ expression was not observed in MCF-7 cells at any time [Fig. 1A]. The increase observed in both TNBC cell lines was blocked by inhibition of neurotrophin interaction with $p75^{NTR}$ [Fig. 1B], using small molecule inhibitors [Y1036 and Ro 08-2750] that block the interaction between $p75^{NTR}$ and its ligands, NGF or BDNF (19) (20) or anti-NGF neutralizing antibody. These data indicate that neurotrophins may regulate the expression of $p75^{NTR}$ in TNBC cells. To confirm

the involvement of neurotrophins in the regulation of $p75^{NTR}$, MDA-MB-231 and HCC1806 cells were screened for expression of $p75^{NTR}$, NGF and BDNF. Both cell lines express *NGFR*, *NGF* and *BDNF* mRNA [Fig. 1C]. An ELISA revealed significantly increased NGF in the culture medium from both MDA-MB-231 and HCC1806 cells during 3 days in culture, although it should be noted that the ELISA would detect both mature NGF and proNGF [Fig. 1D].

Mechanism by which NGF induces an increase in expression of $p75^{NTR}$ in TNBC cells

We next examined the role of transcription/translation and of altered processing in mediating NGF's effect on $p75^{NTR}$ levels, since $p75^{NTR}$ can undergo RIP (16, 21). MDA-MB-231 cells treated with NGF exhibited increased levels of $p75^{NTR}$ protein and mRNA over time [Fig. 2A]. The increase in $p75^{NTR}$ protein could be inhibited by anti-NGF neutralizing antibody [Fig. 2B], and by actinomycin D and cycloheximide [Fig. 2C].

RIP of $p75^{NTR}$ is mediated by the action of α and γ secretases (22). NGF has been shown to induce rapid and robust secretase-dependent proteolysis of $p75^{NTR}$ in PC12 cells (23). To determine the contribution of reduced RIP in the increased levels of FL-$p75^{NTR}$, cells were treated with NGF in the presence or absence of GM6001, an α-secretase inhibitor or DAPT, a γ secretase inhibitor. Epoxomicin was included with the treatments to inhibit proteasomal degradation of the cleavage fragments (24). Constitutive RIP of $p75^{NTR}$ was observed in MDA-MB-231 cells, as seen by the presence of the 19 kDa intracellular domain [ICD] [Fig. 2D, Lane 1]. As expected, both GM6001 and DAPT caused an increase in basal levels of FL-$p75^{NTR}$ and the 25 kDa C-terminal fragment [CTF], respectively [Fig. 2D]. NGF caused a strong reduction in ICD levels, accompanied by increases in FL-$p75^{NTR}$ [Fig. 2D], indicating that NGF elicited RIP inhibition. Furthermore, NGF enhanced the accumulation of FL-$p75^{NTR}$ by GM6001 and DAPT, which was accompanied by reduced CTF accumulation with DAPT.

Neurotrophin-mediated increase in $p75^{NTR}$ promotes apoptosis resistance in TNBC cells

Next we tested the role of $p75^{NTR}$ in the resistance of MDA-MB-231 cells to ceramide-induced cell death. We examined whether the difference in $p75^{NTR}$ levels at different times of culture affected the sensitivity of TNBC cells to cell death induction. HCC1806 cells were used and apoptosis induction by C2-ceramide and sensitivity to Ro 08-2750 was compared at 24 and 72 h, at which times the HCC1806 cells express no or high levels of $p75^{NTR}$, respectively [Fig. 1A]. The cells exhibited a pronounced differential sensitivity to apoptosis

induction at 24 and 72 h, as determined by DEVDase activity and analysis of nuclear morphology [Fig. 3A, B]. Furthermore, Ro 08-2750 treatment caused an enhanced loss of mitochondrial transmembrane potential in MDA-MB-231 cells treated with C2-ceramide [Fig. 3C]. Knock-down of $p75^{NTR}$ using shRNA, sensitized MDA-MB-231 cells to C2-ceramide induced apoptosis, as determined by subG1 analysis [Fig. 3D].

$p75^{NTR}$ expression correlates with neurotrophin expression in basal-like breast cancer subtype

These data provide a link between elevated $p75^{NTR}$ levels and NGF. To examine whether there is a correlation between *NGF* and *NGFR* in patients, gene correlation analysis was carried out using bc-GenExMiner v3.1 which computes correlation coefficients between two genes (25). A weak but highly significant [$p<0.0001$] positive correlation between *NGFR* and *NGF* in BC was found [Fig. 4A], and in certain subtypes including basal-like, ER negative and ER positive BCs [Fig. 4B-D]. In contrast, there was no correlation between *NGFR* and *NGF* in Luminal A, Luminal B or HER2-overexpressing BCs [Fig. 4E-G].

Discussion

Here we show that NGF is secreted by TNBC cells and induces increased levels of FL-$p75^{NTR}$ in these cells. As a consequence, this alters their sensitivity to induction of apoptosis upon NGF inhibition. After 72 h in culture, when $p75^{NTR}$ levels were high, HCC1806 cells exhibited increased sensitivity to apoptosis upon NGF-$p75^{NTR}$ inhibition which was not observed at 24 h when $p75^{NTR}$ levels were low. While it is known that NGF can promote survival of TNBC cells through $p75^{NTR}$ dependent signaling, this is the first report showing that NGF can also upregulate $p75^{NTR}$ expression in BC cells. It is likely that the effect is due to NGF and not proNGF, since treatment of cells with proNGF induced the opposite effect, namely a decrease in $p75^{NTR}$ levels (data not shown). In human disease the expression of *NGF* and *NGFR* mRNAs are positively correlated in several subtypes of BC, including TNBC and basal-like BC. Together these results suggest that NGF can promote survival of TNBC cells through upregulation of $p75^{NTR}$ expression, in addition to its pro-survival signaling through $p75^{NTR}$.

Our results showing that TNBC cell lines secrete appreciable amounts of NGF, support and confirm previous reports that BC cells secrete NGF (11, 26). Widespread expression of NGF in primary breast tumors has been reported (10) and furthermore, a recent study showed that

expression of NGF predicts poor survival of BC patients (27). In an MDA-MB-231 xenograft model, targeting NGF with anti-NGF antibodies or siRNA reduced tumor growth with a corresponding increase in apoptosis, underscoring the importance of NGF to BC (10).

Regulation of expression of $p75^{NTR}$ has been studied extensively over the last decade. For example, $p75^{NTR}$ expression is induced in neurons after seizure (13, 28), ischemia (15, 29) and upon hypo-osmolar stress (14, 30, 31). Exogenous NGF can induce $p75^{NTR}$ expression in prostate cancer cell lines (17). However, to our knowledge this is the first report showing that NGF can regulate $p75^{NTR}$ in BC cells. It achieves increased $p75^{NTR}$ partially through altered transcription/translation and partially through inhibition of RIP, producing increased levels of full length $p75^{NTR}$. NGF transcriptional upregulation of $p75^{NTR}$ could be mediated through early growth response [Egr] and SP1, both of which can bind and transactivate the $p75^{NTR}$ promoter (30, 32), and both of which are activated by NGF (33, 34).

Recently, it has been reported that processing of $p75^{NTR}$ [stimulated by its overexpression using an inducible system] can exert an anti-apoptotic effect in MDA-MB-231 breast cancer cells that is mediated by the CTF (21). In contrast, we show here that although NGF induces increased levels of $p75^{NTR}$ there is a concomitant reduction in its processing; suggesting that NGF inhibition of $p75^{NTR}$ processing may contribute to the increased levels of the full length protein. Since there is also enhanced resistance of the cells to apoptosis, it further suggests that $p75^{NTR}$ processing is not required for the pro-survival signaling. In contrast, other reports have shown that NGF can induce proteolytic processing of $p75^{NTR}$ in PC12 cells (35). This effect required activation of TrkA which led to metalloprotease-mediated shedding of $p75^{NTR}$ and subsequent RIP. It is unclear why TNBC cell lines used in the present study responded in an opposite way, despite the common requirement for TrkA activation in both cell types. Indeed, other studies have recently shown that $p75^{NTR}$ undergoes constitutive processing in BC cells (36). However, the effect of NGF on this process was not examined.

We were interested to know the relevance of our observation that NGF can induce expression of $p75^{NTR}$ to human disease. A correlation analysis of expression of *NGF* and *NGFR* mRNA in patients with BC revealed a significant positive correlation between the expression of the two genes in all BC samples and in certain subtypes, namely basal-like BC, TNBC and ER positive BC. There is considerable overlap between basal-like BC and TNBC (37), and interestingly, basal-like BC has been linked to high expression of $p75^{NTR}$ (38). Together these observations support the hypothesis that NGF may regulate the expression of $p75^{NTR}$ in these

BC subtypes. TNBC, which constitute 15-20% of BCs, exhibit an aggressive phenotype with poor prognosis, mainly due to the lack of targeted therapies (4). Currently, the main treatments for this type of BC are conventional cytotoxic chemotherapy (39). However, anthracyclines can produce unwanted side effects, e.g., doxorubicin can cause myelosuppression and cardiomyopathies (40, 41) Thus, targeting NGF may be a novel therapeutic strategy that could benefit patients with this type of BC.

In conclusion, the data suggest a role for NGF in upregulating the expression of $p75^{NTR}$ in BC. This, in addition to the known pro-survival effect of NGF-$p75^{NTR}$ signaling in BC, support NGF as a good target for BC treatment, especially where targeted therapies are lacking, such as in TNBC and basal-like BC. Anti-NGF therapy holds the possibility of increasing the effectiveness of cyto/genotoxic drugs used as adjuvant therapies in BC treatment.

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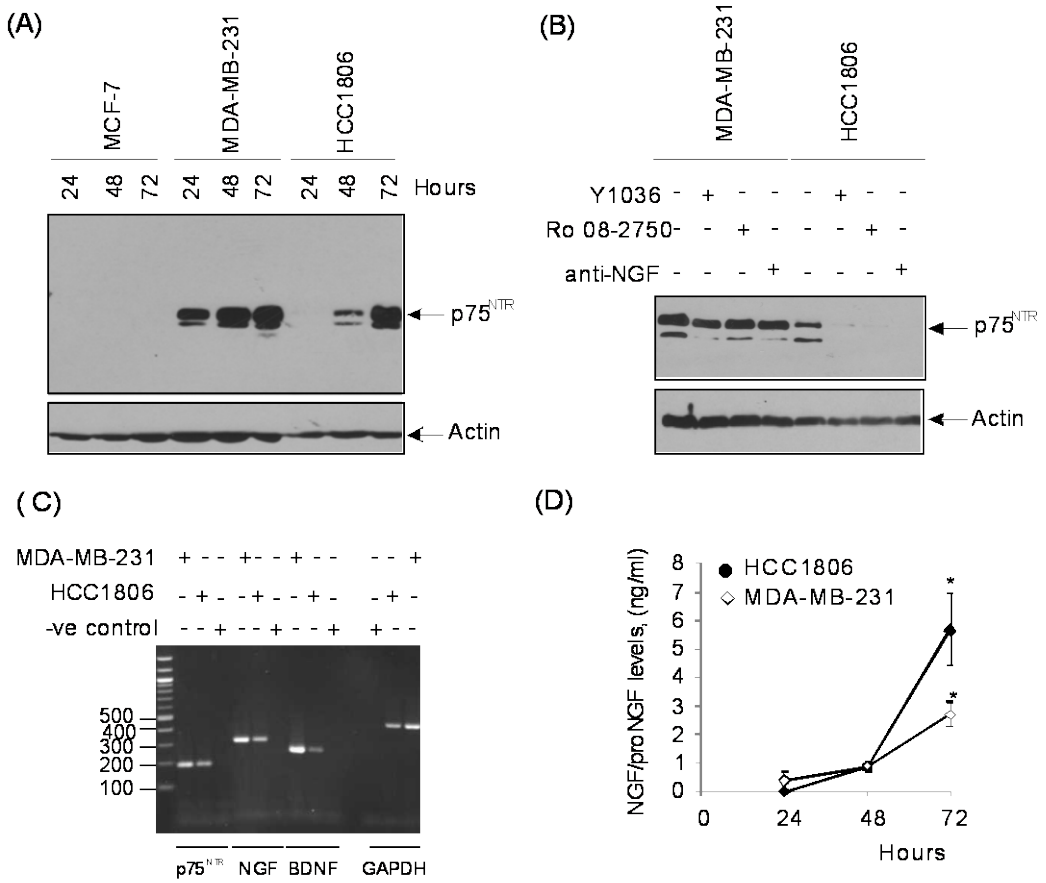

Figure 1 Chakravarthy et al

Fig 1: Neurotrophins mediate increased expression of p75^{NTR} during in vitro culture of TNBC cells [A] MCF-7, MDA-MB-231 and HCC1806 cell lines were harvested at 24, 48 and 72 h and analyzed by immunoblotting using anti-p75^{NTR}. [B] MDA-MB-231 and HCC1806 cell lines were treated with or without 50 µM Y1036, or 2 nM Ro 08-2750, or 2 µg/ml α-NGF 24 h post-seeding and harvested 24 h post-treatment. Whole cell extracts were analyzed by immunoblotting using anti-p75^{NTR}. Actin was used as a loading control. Data are representative of n=3. [C] Total RNA was extracted using Trizol method and mRNA expression of p75^{NTR} [201 bp], NGF [313 bp] and BDNF [266 bp] was carried out with specifically designed primers using RT-PCR. GAPDH [452 bp] was used as a loading control. [D] An ELISA for NGF was carried out on culture medium harvested at 24 h, 48 h and 72 h. Commercially available hβNGF was used as a standard for quantification of NGF in the medium. n=3.

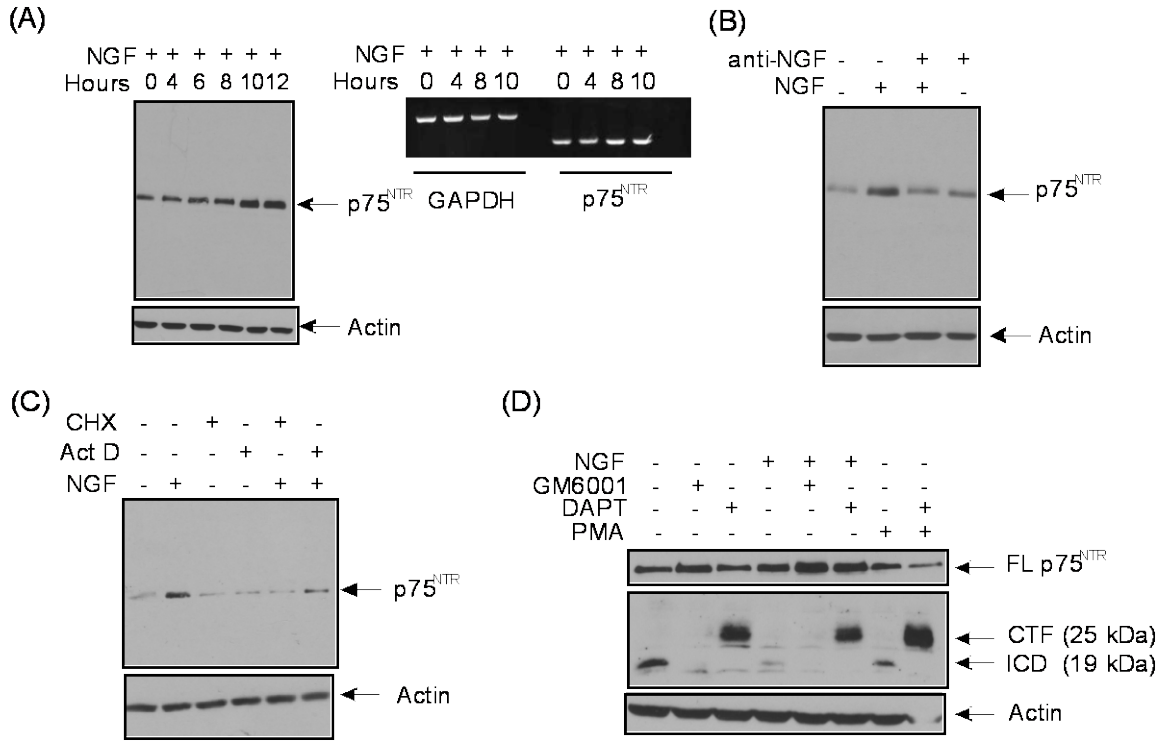

Figure 2 Chakravarthy et al

Fig. 2: Mechanism by which NGF induces an increase in expression of p75^{NTR} in TNBC cells MDA-MB-231 cells were treated with [A] 100 ng/ml mature NGF and were harvested for immunoblotting and for RT-PCR, [B] 100 ng/ml NGF and/or 2 μg/ml NGF-neutralizing antibody, [C] 10 μg/ml actinomycin D or 10 μg/ml cycloheximide for 1 h prior to treatment with or without 100 ng/ml NGF, [D] 1 μM epoxomicin and 10 μM GM6001 or 10 μM DAPT for 1 h before treatment with 100 ng/ml NGF or 300 ng/ml phorbol myristate acetate [PMA], a positive control for activation of secretases. Cells were harvested after 12 h treatment and analysed by immunoblotting using anti-p75^{NTR}. Actin was used as a loading control. Data are representative of n=3.

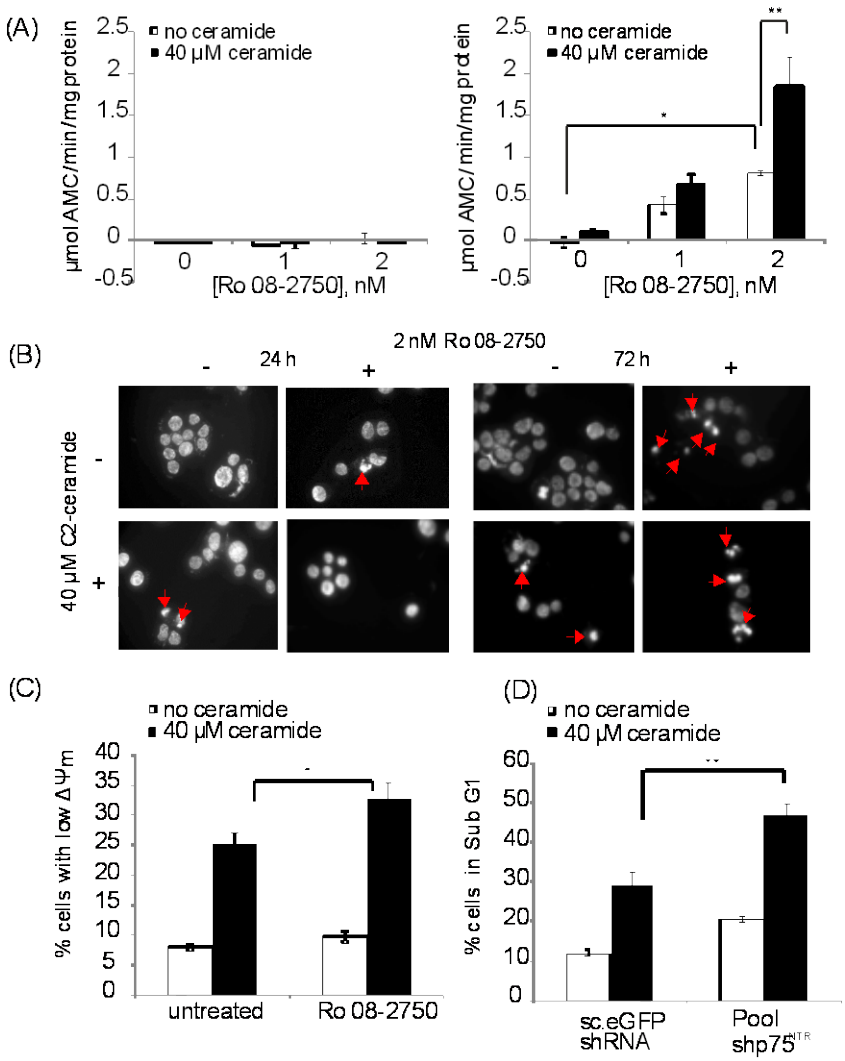

Figure 3 Chakravarthy et a

Fig 3: Neurotrophin-mediated increase in p75^{NTR} promotes apoptosis resistance in TNBC cells HCC1806 cells were treated with 2 nM Ro 08-2750 at 4 or 48 h [graph on the left or right] post-seeding and 1 h after Ro 08-2750 treatment, cells were treated with 40 µM ceramide for 24 h. Cells were harvested for [A] DEVDase assay and [B] DAPI staining. [C] MDA-MB-231 cells were treated with 1 nM Ro 08-2750 for 1 h prior to treatment with 20 µM ceramide for 24 h. Cells were harvested for TMRE assay. [D] MDA-MB-231 cells were transfected with 1 µg shp75^{NTR} and sc eGFP plasmid DNA. At 24 h post-transfection, cells were treated with or without 40 µM ceramide for 24 h and harvested for subG1 analysis. *p<0.05, **p<0.01 and ***p<0.001.

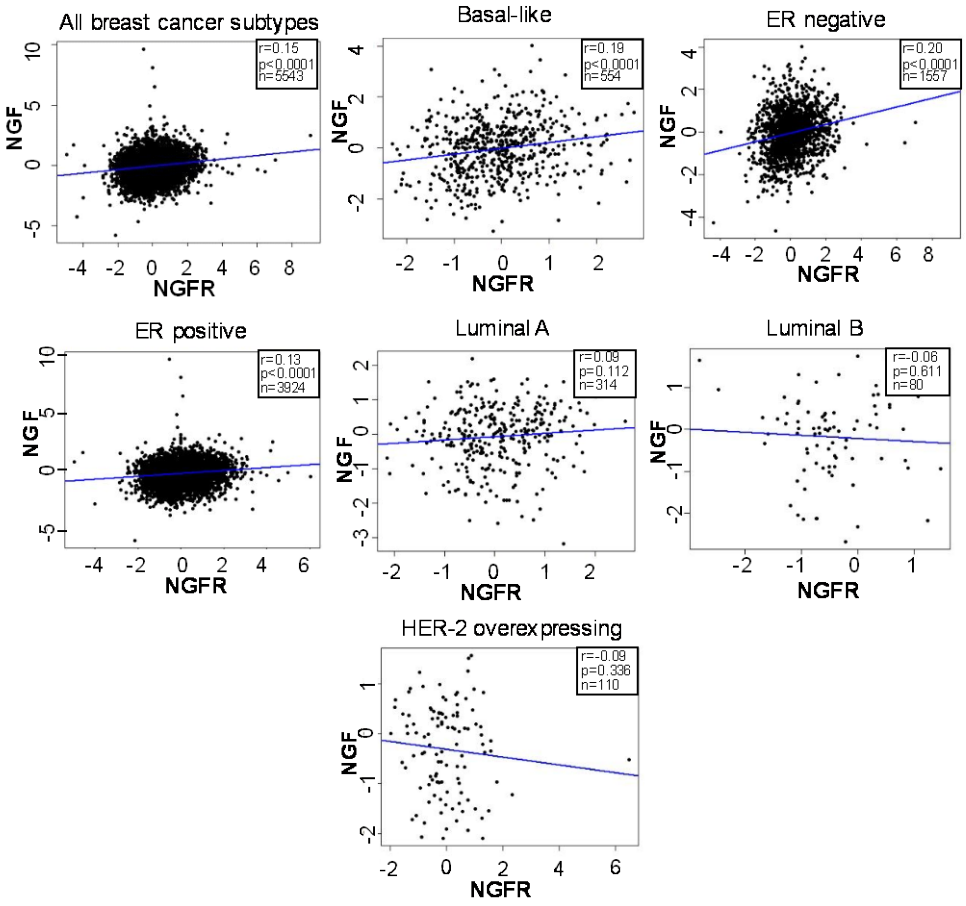

Figure 4 Chakravarthy et al

Fig 4:p75^{NTR} expression correlates with neurotrophin expression in basal-like breast cancer subtype Pearson's pairwise correlation of *NGF* versus *NGFR* was carried out using bc-GenExMiner v3.1. Correlation plots of *NGF* vs *NGFR* in [A] all BC samples, [B] basal-like BC, [C] ER-negative BC, [D] ER-positive BC, [E] Luminal ABC, [F] Luminal B BC and [G] HER2-overexpressing BC.