p75 Neurotrophin Receptor Cleavage by α- and γ-Secretases is required for Neurotrophin mediated proliferation of Brain Tumor Initiating Cells.

Peter A Forsyth 1,2,3*, Niveditha Krishna1, Samuel Lawn3, J Gerardo Valadez 4, Xiaotao Qu 5, David A Fenstermacher 5, Michelle Fournier 6, Lisa Potthast 1, Prakash Chinnaiyan 2,7, Geoffrey T Gibney 2,8, Michele Zeinieh 9, Philip A Barker 9, Bruce D Carter 10, Michael K Cooper 4,11 and Rajappa S Kenchappa 1,2,12,*.

1 Department of Neuro-Oncology, Moffitt Cancer Center and Research Institute and 2Department of Oncological Sciences University of South Florida, Tampa, FL 33612.
3 Tom Baker Cancer Centre, Southern Alberta Cancer Research Institute, University of Calgary, Calgary, Canada.
4 Department of Neurology, Vanderbilt University Medical School, Nashville, TN 37232.
Department of 5 Biomedical Informatics, 6Tissue Core, 7Radiation Oncology and 8Cutaneous Oncology, Moffitt Cancer Center and Research Institute, Tampa, FL 33612.
9 Center for Neuronal Survival, Montreal Neurological Institute, McGill University, Montreal, Quebec H3A 2B4, Canada.
10Department of Biochemistry, Vanderbilt University Medical School, Nashville, TN 37232.
11Neurology services, Veterans Affairs TVHS, Nashville, TN-33212,
12Blanchette Rockefeller Neurosciences Institute and Department of Biochemistry, West Virginia University, Morgantown, WV 26505.

Running title: p75NTR Regulates BTIC proliferation

*To whom correspondence should be addressed: Peter A. Forsyth, M.D., and Rajappa S. Kenchappa, PhD, Department of Neuro-Oncology, Moffitt Cancer Center and Research Institute 12902, Magnolia Drive, Tampa, FL 33612, USA. Tel: +1-813-745-3063; Fax: +1-813-745-3510
Email: Peter.Forsyth@Moffitt.org; Rajappa.Kenchappa@Moffitt.org

Key words: Brain Tumors; Glioblastoma; Cancer Stem Cells; Cell Proliferation; Neurotrophins; Nerve Growth Factor; p75 Neurotrophin Receptor.

Background: p75 Neurotrophin receptor (p75NTR) is an important mediator of invasion of malignant gliomas, but its role in glioma proliferation is unknown.

Results: p75NTR mediates proliferation of Brain Tumor Initiating Cells (BTICs) via cleavage and release of intracellular domain.

Conclusion: p75NTR also regulates proliferation of BTICs.

Significance: p75NTR is a potential target for the treatment of malignant gliomas.

ABSTRACT

Malignant gliomas are highly invasive, proliferative, and resistant to treatment. Previously, we have shown that p75NTR is a novel mediator of invasion of human glioma cells. However, p75NTR’s role in glioma proliferation is unknown. Here we used Brain Tumor Initiating Cells (BTICs), and show that BTICs express neurotrophin receptors (p75NTR, TrkA, TrkB and TrkC), their ligands (NGF, BDNF and NT3), and secrete NGF. Down regulation of p75NTR significantly decreased BTICs proliferation. Conversely, exogenous NGF, stimulated BTIC proliferation through α- and γ-secretase mediated p75NTR cleavage and release of its intracellular domain. In contrast, overexpression of the p75NTR-ICD induced proliferation. Interestingly, inhibition of Trk signaling blocked NGF stimulated BTIC proliferation and p75NTR cleavage; implicate the Trks role in p75NTR signaling. Further, blocking p75NTR cleavage attenuated Akt activation in BTICs, suggesting role of Akt in...
p75NTR mediated proliferation. We also found that p75NTR, α-secretases and the 4 subunits of the γ-secretase enzyme were elevated in GBM patients. Importantly, the ICD of p75NTR was commonly found in malignant glioma patient specimens suggesting that the receptor is activated and cleaved in patient tumors. These results suggest that these might be clinically relevant and p75NTR proteolysis is required for BTIC proliferation and is a novel potential clinical target.

INTRODUCTION

Malignant gliomas remain largely incurable with a poor prognosis (1). Glioblastoma multiformes (GBMs) are highly proliferative, invasive, resistant to treatment, and patients have an average survival of ~1 year (2-4). How malignant gliomas arise is not clear; genetic characterization of GBMs has identified four important signaling pathways including the p53, the retinoblastoma protein, receptor tyrosine kinase and NF1 pathways (5). Mutations in IDH1 are uncommon in primary GBM (<5%) but more common in grade II/III gliomas and in secondary GBMs (6, 7). Isolation of glioma stem cells (GSCs), which we designate as Brain Tumor Initiating Cells (BTICs), from patient tumors suggest that gliomas may arise from these cells (8, 9). GSCs share several features with neuronal stem cells including expression of neuronal stem cell markers (10, 11) and the capability to self-renew and to undergo multilineage differentiation (12-14). GSCs develop tumors when transplanted into immune-deficient mice that phenotypically and molecularly resemble MGs in patients (8, 14). These are relatively resistant to radiation and chemotherapies (15-17) suggesting their role in tumor progression and treatment resistance.

We recently used an unbiased in vivo selection strategy to identify genes required for glioma invasion (18) and found that p75 Neurotrophin Receptor (p75NTR) was upregulated in the highly invasive glioma cells. p75NTR over expressing cells were more migratory and invasive in vitro and in vivo, and receptor proteolysis of p75NTR was required for glioma invasion (19).

The p75NTR is a multifunctional signaling protein that regulates a variety of biological effects which are highly cell-type and context specific. Its effect ranges, from neurite outgrowth to myelin formation to cell survival and death (20, 21). p75NTR is also implicated in regulating proliferation and differentiation of neuronal and non neuronal cells (22, 23). Importantly, p75NTR mediates neuronal proliferation and differentiation when bound by BDNF during neurogenesis (24). However, the role of p75NTR and its signaling pathways in glioma stem cell proliferation is unknown. Here, we show that p75NTR is required for glioma stem cell proliferation, and this function is associated with Trk-dependent p75NTR proteolysis by α-secretase and γ-secretases, which releases its intracellular domain and ultimately triggers proliferation. Further, p75NTR, α-secretase and γ-secretase cleavage products are elevated in malignant glioma tumor specimens suggesting this may be relevant clinically and target two hallmarks of cancer (invasion and proliferation).

EXPERIMENTAL PROCEDURES

Brain Tumor Initiating Cell culture

In this study, we used four different glioma stem cell lines/BTICs isolated from human GBMs (54, 31 and G144) and a giant cell GBM (G179). Lines 54 and 31 were isolated by Dr. Michael Cooper (Vanderbilt University). Lines G144 and G179 were generated by Dr. Peter Dirks (The Hospital for Sick Children; 14), deposited in the Biorep cell bank. We then purchased these lines from Biorep cell bank, Milano, Italy [catalog # NS00013*A (G144), catalog # NS00011*B (G179)]. BTICs were cultured under adherent conditions on Laminin (Sigma, catalog # L2020) coated plates in serum free neuronal stem cell media (Stem cell technology) supplemented with 1% N2 (containing Insulin, Apo-transferrin, BSA, Progesterone, Putrescine, and Sodium selenite) and 1% B27 (Invitrogen), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 ng/mL of FGF and EGF (Stem cell technology) as described previously (14). BTICs were supplied with new media every 4-5 days and were dissociated using Accutase (Sigma, catalog # A6964).
U251 glioma cell lines stably expressing p75NTR were cultured in Dulbecco’s modified Eagle’s medium DMEM (Invitrogen, Gaithersberg, MD) containing 10% FBS, 2 mM L-glutamine, 1mM sodium pyruvate, 100 µg/ml streptomycin, 100U/ml penicillin and 400 µg/ml of G418.

**Total RNA isolation, cDNA synthesis, RT-PCR analysis and Quantitative PCR.**

Total RNA was isolated from BTICs using an RNeasy mini kit (Qiagen, catalog # 74104) per the manufacturer’s protocol. Total RNA (1 µg) was reverse transcribed into cDNA using SuperScript VILO cDNA synthesis kit (Invitrogen, catalog # 12532) and specific primer sets against human NGFR/p75NTR (Qiagen, cat# QT00056756, NM_002507, 118 bp), NTRK1/TrkA (Qiagen, catalog # QT00054110, NM_002529, 112bp), NTRK2/TrkB (Qiagen, catalog # QT00082033, NM_006180, 103 bp), NTRK3/TrkC (Qiagen, catalog # QT00052906, NM_002530, 143 bp), NGF (Qiagen, catalog # QT00001589, NM_002506, 73 bp), BDNF (Qiagen, catalog # QT00235368, NM_001143805, 120 bp), NT3 (Qiagen, catalog # QT00204218, NM_001102654, 104 bp) and human Actin B (Qiagen, calatog # QT01680476, NM_001101, 104 bp). PCR reaction conditions were: 5 min 95°C for initial PCR activation, 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min, and 72°C for 10 min for final extension. The same amount of human brain total RNA (Clontech, catalog # 636530) for cDNA synthesis and PCR reactions used as a positive control. After amplification PCR products were run on a 2% agarose gel and gel images were captured for further analysis. Quantitative PCR was conducted with Real-time PCR detection system (Biorad) using cDNA, SYBR green PCR master mix (Applied Biosystem, catalog # 4309155), and primers for TrkA, (Qiagen, catalog # QT00054110, NM_002529, 112bp), ADAM17 (Qiagen, catalog # QT00055580, NM_003183, 109 bp) and Actin (Qiagen, calatog # QT01680476, NM_001101, 104 bp). All samples were run in triplicates, data was analysed at appropriate cycle number, and expression was calculated as ∆∆Ct and normalized to Actin.

**Transfection of Brain tumor initiating cells**

BTICs were dissociated using Accutase as previously described (14). The cell suspensions were then transfected with Stealth control-siRNA (Invitrogen, catalog # 452001) or Stealth siRNAs to p75NTR with p75NTR duplex siRNA with following sequence, p75NTR-siRNA#1, sense sequence: CACUUCUGACCACACUUCGUGUCCA, antisense sequence: AAAUUAUUACCCAGACUCUGUCC.

p75NTR-siRNA#2, sense sequence: GGACAGAGUCUGGGUGUAUUU, antisense sequence: AAAUUAUUCCACCCAGACUCUGUCC. Cells were transfected with 40 nmoles of p75NTR-siRNA#1 or p75NTR-siRNA#2 or control-siRNA by using Amaxa electroporation kit (Lonza, catalog # VPG-1004) and T-030 program on an Amaxa electroporation device. For Western blotting analysis, three days after electroporation, cells were lysed and used for p75NTR Western blotting. For proliferation assays, cells were used four days after electroporation, for MTT assays cells were washed and added with MTT reagent and lysed, for Tryphan blue assay cells were collected added with Tryphan blue and for immunostaining cells were fixed and immunostained with Ki67 antibody.

In some of the experiments, the BTICs were transfected with 2 µg of wild type-p75NTR, or γ-secretase resistant mutant-p75NTR (p75FasTM) (25) (kindly provided by Dr. Moses V. Chao (Skirball Institute, New York University) using Amaxa electroporation method as described above. Three days after the electroporation, cells were treated with the proteosome inhibitor epoxomycin (1 µM, Cabiochem, catalog # 324800) alone or along with 100 ng/ml of NGF (Harlan, catalog # BT3061) for 6 hr, and then cells were lysed and subjected to p75NTR Western blotting. For assessing proliferation, 48 hr after transfection cells were treated with 100 ng/ml of NGF or left untreated for three days, then fixed using 4% paraformaldehyde, stained for Ki67, and Ki67 positive cells were scored for proliferation.

In some of other experiments, BTICs were electroporated with control-siRNA, p75NTR-
SiRNAs 1 and 2 or p75FasTM as described above. Cells were maintained in neurobasal media without EGF and FGF for 48 hr, and then cells were switched to media containing EGF and FGF for 6 hr, lysed and subjected to p75NTR, phospho-Akt (1:1000, Cell signaling, catalog # 4056) and Actin (1:1000, Cell signaling, catalog # 4967) Western blotting analysis. BTICs were also electroporated with GFP alone or with GFP and p75NTR-intracellular domain (p75NTR-ICD) together (kindly provided by Dr. Philip Barker, McGill University, Canada) and 2 days later cells were lysed and performed p75NTR-ICD and Tubulin Western blotting. For examining the proliferation, 3 days following transfection cells were fixed and stained with Ki67 antibody.

**Western blotting analysis**

BTICs were cultured under neuronal stem cell media as described above, then cells were harvested, lysed in RIPA buffer (10 mM tris-HCl, 1 mM EDTA, 0.4 mM EGTA, 0.1% SDS, 140 mM sodium chloride, 0.1% sodium deoxycholate, 1% triton X-100, and added with 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, aprotinin and leupeptin) and lysates were used for p75NTR (1:3000, provided from Dr. Bruce Carter, Vanderbilt University, USA), TrkB (1:1000, Cell signaling, catalog # 4603), TrkC (1:1000, Cell signaling, catalog # 3376) and Tubulin (1:1000, Calbiochem, catalog # CP06) Western blotting. In some experiments, to detect the intracellular domain (ICD) of the receptor, cells were washed and treated with the proteosome inhibitor epoxomycin. Epoxomycin (1 µM, Calbiochem, catalog # 324800) was added to cells with or without γ-secretase inhibitor DAPT (200 nM, Calbiochem, catalog # 565770) or metalloprotease inhibitor TAPI-2 (500nM, Calbiochem, catalog # 579052) or Trk inhibitor K252a (200 nM, Sigma, catalog # K2015) in the presence or absence of 50-100 ng/ml of NGF (Harlan, catalog # BT3060) to activate p75NTR. Then cell were lysed in RIPA buffer and lysates were subjected to p75NTR Western blot analysis (1:3000 of p75NTR antibody raised against intracellular domain) as described previously (26, 27). The lysates were collected following transfection of various p75NTR constructs were also subjected to p75NTR-ICD Western blotting. These blots were reprobed for the loading control Tubulin and Actin (1:1000, Cell signaling, catalog # 4967).

**Enzyme-linked immunosorbent assay for neurotrophins**

BTICs 54, 31, G144 and G179 were maintained in neuronal stem cell media for 5 days, then medium was collected and filtered through a syringe filter and used for measuring neurotrophins by ELISA method. ELISA was performed for NGF (Human NGF ELISA kit, Boster Immunoleader, catalog # EK0469), BDNF (Human BDNF ELISA kit, Boster Immunoleader, catalog # EK0307) and NT3 (Human Neurotrophin-3 ELISA kit, Boster Immunoleader, catalog # EK0472) according to manufacturer’s instructions. Data is expressed as ng of neurotrophins secreted/ml of media.

**MTT and Trypan blue assay for cell proliferation/cell viability**

Cell proliferation or cell viability of BTICs was measured using a MTT assay. BTICs were dissociated and transfected with control-siRNA or p75NTR-siRNAs as described above and then plated on 24 well plates coated with laminin. Four days after transfection, cells were washed with 1X PBS and then incubated with 240 µl of MTT solution (1 mg/ml, Sigma) at 37°C incubator. 2 hr later MTT solution was removed, and cells were lysed adding 240 µl of isopropanol containing 0.04 M HCl and 160 mM NaOH, and incubated for 10 min at room temperature. Then, absorbance was measured at 570 and 630 nm. Each sample was run in triplicate, and the data were presented as a percentage of control.

For Trypan blue assay, cells were collected in eppendorf tube, added with equal volume of 0.4% Trypan blue solution (Sigma, catalog # T8154) and measured the total number of live cells using TC10™ Automated cell counter (Biorad). Data was expressed as total number of cells/ml.

**Ki67 immunostaining for cell proliferation**

BTICs were transfected with control-siRNA, p75NTR-siRNA, wildtype-p75NTR, cleavage resistant mutant-p75NTR, and cells treated with DAPT (200 nM) or TAPI-2 (500 nM) or K252a (200 nM) and with NGF (25, 50 and 100 ng/ml), BDNF (50 ng/ml, Peprotech, catalog #
NT3 (50 ng/ml, Millipore, catalog # GF031), anti-NGF (0.1 µg/ml, Millipore, catalog # MAB5260Z) or control-IgG (0.1 µg/ml, Calbiochem, catalog # N101) were fixed in 4% paraformaldehyde and processed for the proliferative marker Ki67 by immunostaining. Cells were permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 10 min at room temperature, washed with 1XPBS and blocked with 10% normal goat serum for 1 hr at room temperature. Then cells were incubated with the Ki67 antibody (1:100 dilution, Biocare medical, catalog # CRM 325C) in PBS containing 0.1% Triton X-100 overnight at 4°C, this is followed by incubation with anti-rabbit Alexa 488 (1:500 dilution, Molecular probes, catalog # A11008) or anti-rabbit Alexa 546 (1:500 dilution, Molecular probes, catalog # A11010) in 1XPBS for 1 hr at room temperature and stained with DAPI (Vector labs) to visualize nuclei. Cells were viewed and captured using LSM 710 Meta confocal microscope (Zeiss, Germany) at 40x. The number of Ki67 positive (mainly nuclear) cells was quantified by examining the total number of cells counted (by DAPI staining) and proliferation was expressed as a percentage.

Bioinformatics analysis
Gene expression analysis from two different datasets was used in this study. One contains microarray expression from The Cancer Genome Atlas (TCGA) data portal and level 3 expression data on Affymetrix U133A chip was used in this analysis. The other dataset contains microarray expression data for 108 GBM patients from Moffitt’s Total Cancer Care (TCC) program. A customized affymetrix chip was used to generate the expression profile and MAS5 was used to normalize the expression data. R package is used to analyze gene expression data in which gene expression level was categorized into 3 groups: up-regulated, intermediate and down-regulated according the quartile of normalized expression data.

Tumor samples
Malignant glioma tumor specimens were obtained from patients who were either previously irradiated or newly diagnosed. Samples were immediately snap frozen and graded according to the World Health Organization (WHO). Tumor samples were used to isolate total RNA for PCR analysis of p75NTR and NGF, for Western blotting samples were lysed in RIPA buffer, debris removed by centrifugation and lysates were subjected to Western blotting using p75NTR-ICD and Tubulin antibodies as described above.

RESULTS
Neurotrophin receptors and ligands are expressed in Brain Tumor Initiating Cells.
Neurotrophins and their receptors (p75NTR, TrkA, TrkB, TrkC) play important roles in regulating cell survival and proliferation in multiple cell types (20, 21, 28). p75NTR signaling is required for survival and proliferation of breast cancer cells (29-31). We used four different patient derived BTICs, designated as 54, 31, G144 and G179, and we investigated whether p75NTR regulates their proliferation. We cultured BTICs on laminin coated plates with EGF and FGF (14) and confirmed they express the neuronal stem cells markers Sox2 and Nestin (14 & data not shown). RT-PCR analysis demonstrated that all four BTICs express the neurotrophin receptors p75NTR, TrkB and TrkC, TrkA was expressed only in G179 (Figure 1A and 1B). However, quantitative PCR analysis data demonstrated the expression of TrkA in all BTICs examined (Figure 1C). Western blotting of p75NTR, TrkB and TrkC shows BTICs express neurotrophin receptor proteins (Figure 1D, 1E and 1F). For TrkB and TrkC with the antibodies we used (Cell signaling, 80E3, catalog # 4603 for TrkB and C44H5, catalog # 3376 for TrkC) as anticipated, we detected bands at 140 kDa for full length receptors and additional bands at 90 kDa for truncated forms of the receptors (Figure 1E and 1F). However, expression patterns of TrkB and TrkC were different in each BTICs. These data indicate that neurotrophin receptors are widely expressed in BTICs.

Mammalian Neurotrophins, such as nerve growth factor (NGF), brain derived neurotrophic factors (BDNF), and others, can be expressed in cancer cells. Neurotrophins and their receptors play a critical role in tumor growth and proliferation. In this study, we investigated the expression and role of neurotrophin receptors (p75NTR, TrkA, TrkB, TrkC) in four patient-derived BTICs. We found that all four BTICs expressed p75NTR, TrkB and TrkC, while TrkA was only expressed in one of the BTICs, G179. Additionally, RT-PCR and Western blotting analyses confirmed the expression of these receptors in BTICs. These results suggest that neurotrophin receptors may play a role in regulating proliferation and survival of BTICs.
factor (BDNF) and neurotrophin 3 (NT3) are secretory proteins that regulate neuronal survival and growth by binding to Trks and the p75NTR. NGF exhibits specific affinity for TrkA receptor, BDNF for TrkB receptor, and NT3 for TrkC receptor. However, every neurotrophin binds to p75NTR with equal affinity (21). Therefore, we hypothesized that BTICs express and secrete neurotrophins. RT-PCR analysis showed that neurotrophic factors NGF, BDNF and NT3 are expressed in all BTICs we examined (Figure 1G).

Estimation of secreted neurotrophins in culture media by ELISA demonstrated that all neurotrophins were secreted into the media, however, NGF (22.40 ng/ml) secretion was approximately 10 fold higher than BDNF (2.33 ng/ml) and NT3 (2.50 ng/ml) secretion (Figure 1H). These results show that neurotrophins are secreted by BTICs and may activate receptors in an autocrine and/or paracrine fashion to mediate BTIC invasion, survival and proliferation.

**Knock down of p75NTR decreases brain tumor initiating cells proliferation.**

Since we have previously shown that p75NTR mediates glioma invasion (18, 19). We wanted to determine if it mediated other aspects of the malignant phenotype such as proliferation. To address this we used siRNAs to down regulate p75NTR expression in BTICs. We transfected BTICs with control-siRNA or two different p75NTR-siRNAs, then three days later confirmed the knock down of p75NTR protein by Western blotting (Figure 2A and 2B). We then assessed the effects of p75NTR knock down on proliferation. First we used the MTT and tryphan blue assay to measure proliferation (32, 33). Knock-down of p75NTR significantly decreased proliferation of all four BTICs as measured by MTT assay (Figure 2C-2F) and trypan blue assay (Figure 2G-2I), suggesting that p75NTR normally facilitates BTIC proliferation.

To confirm the role of p75NTR in BTIC proliferation, we also used Ki67 immunostaining method. Ki67 is a nuclear protein highly linked with cell proliferation and extensively used as a proliferation marker in human tumors (34, 35). Following p75NTR knock down by two p75NTR-siRNAs, there was a decrease in proliferation rate of BTICs in vitro (Figure 3). The decrease in proliferation rate after p75NTR knock down for BTIC-54 was 37.68% and 36.28% (Figure 3A and B), for BTIC-31 was 32.31% and 35.36% (Figure 3C), for BTIC-G144 was 25.22% and 28.83% (Figure 3D), and for G179 was 31.21% and 32.69% (Figure 3E) for p75NTR-siRNA-1 and p75NTR-siRNA-2 respectively. Taken together, these results demonstrate that p75NTR is expressed in patient derived BTICs and is required for BTIC proliferation.

**NGF stimulates BTIC proliferation and this is blocked by NGF blocking antibody.**

p75NTR and TrkA form a high affinity binding complex when they are co-expressed and this increases the binding affinity of NGF to the TrkA receptor and enhances tyrosine kinase activity and survival (36-39). However, p75NTR can also promote survival independently of TrkA and binding of NGF to p75NTR alone supports cell survival through activation of NF-κB (40-42).

All four BTICs we examined secrete 10 fold higher levels of NGF [the ligand for both p75NTR and TrkA] compared to levels of BDNF [the ligand for both p75NTR and TrkB] and NT3 [the ligand for both p75NTR and TrkC, (Figure 1H)]. Therefore, we hypothesized that NGF-mediated activation of p75NTR and TrkA independently, or as a p75NTR-TrkA complex, stimulates BTIC proliferation. NGF has been implicated in tumor cell proliferation and survival (31); since our BTICs secrete approximately 25 ng/ml of NGF, we next examined the effects of multiple concentrations (such as 25, 50 and 100ng/ml) of NGF and anti-NGF on BTIC proliferation. We treated the BTICs with different concentrations of NGF for 3 days and assessed the proliferation by Ki67 immunostaining. Interestingly, NGF at all concentrations significantly enhanced proliferation of BTIC-54 was 24.79% at 25ng/ml, 38.26% at 50 ng/ml and 34.22% at 100ng/ml (Figure 4B), in BTIC-31 was 17.65% at 25 ng/ml, 35.09% at 50 ng/ml and 16.62% at 100ng/ml (Figure 4C), in BTIC-G144 was 10.26% at 25 ng/ml, 33.49% at 50 ng/ml and 27.97% at 100ng/ml (Figure 4D), and in G179 was 28.23% at 25 ng/ml, 39.11% at 50 ng/ml and 31.25% at 100 ng/ml (Figure 4E). In order to examine the effects of endogenously secreted NGF on proliferation, we treated the cells with control-IgG or NGF antibody to sequester
secreted NGF (antibodies were added to every 24 hr). NGF antibody treatment significantly decreased the proliferation of all BTICs with 41.76% decrease in BTIC 54 (Figure 4F), 21.58% in BTIC 31 (Figure 4G), 39.41% in BTIC G144 (Figure 4H) and 43.29% in BTIC G179 (Figure 4I); control-IgG had no effect on proliferation (Figure 4F) suggesting the autocrine and/or paracrine action of NGF in BTIC proliferation. Our results indicate that NGF acts through p75NTR/TrkA to stimulate BTIC proliferation.

BTICs also express TrkB and TrkC receptors (Figure 1B, 1E and 1F) and their ligands BDNF and NT3 (Figure 1G), but the secretion levels of BDNF and NT3 were dramatically lower compared to NGF (Figure 1H). We next examined the effect of exogenous BDNF and NT3 on BTIC proliferation. We treated the BTICs with 50 ng/ml of BDNF and NT3 (BDNF and NT3 was added for every 24 hr), 3 days later these were examined using Ki67 immunostaining. Interestingly, both BDNF (Figure 5A and 5C) and NT3 (Figure 5B, 5D) increased proliferation in all BTICs. These results indicate that all neurotrophins are capable of mediating BTIC proliferation in vitro.

NGF triggers p75NTR cleavage, which is necessary for proliferation of Brain Tumor Initiating Cells.

p75NTR is cleaved by the metalloproteinase, TACE/ADAM17 and produces a soluble extracellular domain (ECD) and the carboxy-terminal fragment (CTF) (43-46, 25, 27). The released CTF can be further processed by γ-secretase within the transmembrane domain, to release the intracellular domain (ICD) into the cytosol (47-49, 26, 27, 50). The released p75NTR ICD generates signals to regulate several physiological functions which are cell-type specific. In cerebellar granular neurons, the ICD inhibits MAG-induced neurite outgrowth (49) and in sympathetic neurons, it mediates proapoptotic ligand-induced cell death (26, 27), mediates Trk receptor mediated cell neuronal survival (46). The p75NTR ICD has also been implicated in cell division and proliferation of spiral ganglion Schwann cells (51). Further, the ICD translocates to the nucleus and modulates gene expression (52).

We have previously shown that p75NTR cleavage and release of the ICD is necessary for receptor-mediated glioma invasion in vitro and in vivo (19). Therefore, we next wanted to determine if NGF induces α- and γ-secretase-dependent p75NTR proteolysis and if this is required for BTIC proliferation.

We first used a pharmacological approach to test whether p75NTR is cleaved by α-secretase and γ-secretase in response to NGF treatment, washed the BTICs (line 54) with fresh media and pre-treated with 500nM of α-secretase inhibitor TAPI-2 or with 200nM of γ-secretase inhibitor DAPT or left them untreated and then added 100ng/ml NGF for 6 hr. The cells were also treated with the proteosome inhibitor epoxomycin (1µM) to enhance detection of the cleaved fragments of the receptor such as the ICD which is quickly degraded by proteosome following its release (48, 46, 27, 19). We found that NGF treatment of the BTICs causes accumulation of the ICD and that DAPT, which blocks the release of ICD, leads to CTF accumulation. TAPI-2 blocks both CTF generation and ICD release (Figure 6A). These data show that NGF treatment leads to α- and γ-secretase-mediated cleavage of the p75NTR in BTICs.

In order to determine whether p75NTR cleavage was required for NGF induced BTIC proliferation we left BTIC lines untreated or exposed them to 500nM of α-secretase inhibitor TAPI-2 or with 200nM of γ-secretase inhibitor DAPT; cells were then treated with 100ng/ml of NGF for three days (DAPT, TAPI-2 and NGF were added to cells for every 24 hr) and the effect of NGF on BTIC proliferation was measured by Ki67 staining. We found that both TAPI-2 and DAPT completely blocked the NGF-mediated stimulation of proliferation (Figure 6B-6I). Somewhat surprisingly, inhibition of α-secretase or γ-secretase reduced proliferation even when unstimulated by exogenous NGF (Figure 6B-6I). These results demonstrate that p75NTR cleavage and release of ICD is necessary to stimulate NGF mediated proliferation of BTICs.

Expression of cleavage resistant mutant p75NTRs prevents NGF stimulated Brain Tumor Initiating Cell proliferation.
Since, α-secretase and γ-secretase are known to cleave many other transmembrane proteins to generate signaling intracellular domains (55), it also likely that inhibitors block the cleavage of other non-p75NTR substrates. To confirm that BTIC proliferation by NGF is due to cleavage of p75NTR and release of ICD by α-secretase, we used cleavage-resistant mutant p75NTR, p75FasTM which is resistant to γ-secretase mediated cleavage, and can block p75NTR dependent apoptosis (26, 27) and invasion of glioma cells (19). BTICs were transfected with wild type-p75NTR or p75FasTM by electroporation or left untransfected; three days later the transfected cells were washed twice with media, treated with NGF and epoxomycin for 6 hr and receptor cleavage was measured. NGF-induced p75NTR cleavage was found in BTICs left untransfected or transfected with wild type-p75NTR whereas in BTICs transfected with p75FasTM, the receptor cleavage and ICD release was completely blocked (Figure 7A). These results demonstrate that mutant p75NTR blocks the processing of wild type p75NTR in BTICs.

To assess whether p75FasTM could block NGF-p75NTR mediated cell proliferation, BTICs were transfected with these constructs by electroporation, and two days after transfection cells were treated with NGF (NGF was added for every 24 hr) for three days. Then we measured the proliferation by Ki67 immunostaining, in cells left untransfected or transfected with wild type-p75NTR, NGF stimulated proliferation; however, the γ-secretase resistant mutant completely blocked the NGF-induced proliferation (Figure 7B-E). These results show that γ-secretase mediated cleavage of p75NTR and release of its ICD is essential for the NGF mediated proliferation of BTICs.

Inhibition of Trk signaling blocks NGF mediated BTIC proliferation and p75NTR cleavage.

NGF is known to signal through both p75NTR and TrkA to mediate physiological functions such as neuronal survival, proliferation and differentiation (21). The BTICs we used in this study express both p75NTR and TrkA, and also secrete NGF (Figure 1), inhibition of secreted NGF decreases BTIC proliferation and treating these cells with exogenous NGF stimulates proliferation (Figure 4). Further, NGF stimulated the α- and γ-secretases mediated p75NTR cleavage, which is necessary BTIC proliferation (Figure 6 and 7). However, the role of TrkA signaling in this context was not known. Recent studies have demonstrated that activation of TrkA receptor with NGF regulates α-secretase and γ-secretase mediated p75NTR cleavage and releases the intracellular domain (56, 50), which is required for NGF mediated cell survival (50, 46). Based on these observations, we hypothesized that NGF binding to the TrkA receptor in BTICs activates signals to mediate p75NTR cleavage which stimulate BTIC proliferation. Therefore, we examined the role of Trk signaling in BTIC proliferation and p75NTR cleavage. We treated BTICs with 200nM of Trk inhibitor K252a or left untreated with or without 50ng/ml of NGF for three days (K252a and NGF were added to cells for every 24 hr) and proliferation was measured by Ki67 immunostaining. Figure 8A-8D show that K252a completely blocked NGF-mediated BTIC proliferation.

To test whether Trk signaling regulates p75NTR cleavage; BTIC line 54 was left untreated or treated with 200nm of Trk inhibitor K252a and then exposed to 50ng/ml NGF or vehicle for 6 hr, in the presence of epoxomycin, K252a blocked accumulation of the CTF and ICD (Figure 8E), indicating that Trk signaling is necessary for p75NTR cleavage in brain tumor initiating cells.

Studies have shown that TACE/ADAM17 is required for release of p75NTR’s extracellular domain (ECD) and the carboxy-terminal fragment (CTF) via its upgulation or phosphorylation at threonine 735 in response to neurotrophin treatment (45, 27, 46). Further, knock down of ADAM17 blocked p75NTR cleavage mediated activation of Jun- kinase and Akt, also cleavage mediated cell death and survival (27, 46). Therefore, we examined the expression levels of ADAM17 in response to NGF in BTICs. Quantitative PCR analysis demonstrates that ADAM17 mRNA was increased by 2.5 fold following NGF treatment (Figure 8F). It is possible that ADAM17 might also be phosphorylated following NGF treatment as...
p75NTR Regulates BTIC Proliferation

reported recently (46). Considering the role of ADAM17 in tumor formation (53, 54), these results suggest that ADAM17 might be involved in neurotrophin mediated BTIC proliferation and tumor development.

Expression of p75NTR’s Intracellular domain (ICD) alone is sufficient to stimulate BTIC proliferation.

In order to determine whether γ-secretase released p75NTR ICD is sufficient to induce BTIC proliferation and to rule out the possible involvement of other substrates of γ-secretase in this process, we expressed the ICD in BTICs by Amaza electroporation and examined the effect on proliferation. Same ICD construct shown induce apoptotic cell death in PC12 cells (57) and in sympathetic neurons (26), and activated Jun kinase (27) and Akt (46) suggesting signaling capabilities of ICD. Expression of the ICD in BTIC line 54 was confirmed 2 days after transfection (Figure 9A) and proliferation was assessed one day later. We found that expression of ICD stimulated the proliferation of brain tumor initiating cells (Figure 9B and 9C). These data clearly show that ICD alone is sufficient to mediate proliferation without involvement from other substrates of the γ-secretase.

p75NTR cleavage is required for Akt activation in Brain Tumor Initiating Cells.

The Akt pathway is necessary for glioma cell proliferation and invasion, and glioma development and progression (58, 59). p75NTR cleavage by α- and γ-secretases has also been implicated in regulating neurotrophin-dependent survival through activation of Akt signaling (50, 46). Therefore, we examined the Akt activation when p75NTR was downregulated and receptor cleavage was blocked. We electroporated control-siRNA, p75NTR-siRNAs (siRNA 1 and 2 combined) to knock down p75NTR or γ-secretase resistant mutant p75NTR (p75FasTM) shown to block endogenous p75NTR cleavage and proliferation (Figure 7), cells were maintained in media without growth factors for 2 days, then replaced with the media with growth factors EGF and FGF for 6 hr. We found that knock down of p75NTR or blocking its γ-secretase dependent cleavage significantly decreased Akt activation (Figure 10A and 10B), suggesting that p75NTR cleavage mediates BTIC proliferation via Akt activity.

p75NTR, α-secretase and γ-secretase components are expressed, and p75NTR cleavage occurs in vivo in malignant glioma patient tumors.

p75NTR is expressed in high grade malignant gliomas (18, 60) indicating its potential role in glioma development and progression in human, however, the mechanisms that underlie this are not known. Several recent studies have shown that p75NTR undergoes regulated intramembrane proteolytic cleavage via an α-secretase and then by a γ-secretase in a manner analogous to Notch, amyloid precursor protein (APP) and Erb4 (48, 47, 55). Further, the p75NTR ICD has signaling potential and inhibits neurite outgrowth in cerebellar granular neurons through the Rho pathway (49), induces ligand dependent cell death in sympathetic neurons via activation of JNK (26, 27), mediates cell survival and growth arrest through Akt activation (50, 46), regulates angiogenesis via stabilization of HIF-1α (61) and also been implicated in cell division and proliferation of spiral ganglion Schwann cells (51). We have also found that p75NTR cleavage was required for glioma invasion in vivo and in vitro (19) and proliferation of glioma initiating cells in vitro (Figure 6 and 7). We therefore hypothesized that α-secretase and γ-secretase enzymes are expressed in human brain tumors and p75NTR cleavage occurs in vivo in human malignant glioma specimens.

We used microarray expression data from the TCGA (Figure 11A), which has gene expression data from 528 GBM patients and 10 normal brain tissues and validated our findings in an independent data set derived from the Moffitt Cancer Center Total Cancer Care (MCC) data set (Figure 11B), which has no expression data from normal brain tissue. We examined the expression levels of p75NTR (NGFR), TrkA (NTRK1), NGF as well as genes related to p75NTR processing such as α-secretases (ADAM10 and ADAM17/TACE) and the γ-secretase enzyme subunits (PSEN1, PSEN2, NCSTN and APH1). In
the TCGA data p75NTR, α-secretase and γ-secretase enzymes levels were elevated in GBM tissue compared to normal brain tissue (Figure 11A), and MCC data set showed a similar pattern of expression (Figure 11B). However, TrkA levels were not expressed at higher levels than controls (Figure 11A). These results indicate that increased expression of p75NTR and its cleavage related signaling components are present and elevated in the tumors derived from patients with GBMs and our observations above are not an artifact of tissue culture conditions.

In order to both validate the expression data at the level of RNA and protein expression and to determine directly if p75NTR is cleaved in malignant glioma tumor specimens we examined snap frozen tumor specimens from a subset of patients with malignant gliomas. We used tumors from glioma patients (ranging from WHO grade I to grade IV tumors) and performed RT-PCR analysis for p75NTR and NGF using human specific primers, and p75NTR Western blotting using an antibody raised against the CTF and ICD of the receptor. NGF and p75NTR were expressed in glioma tumors (Figure 11C). CTF and ICD fragments were found in every tumor we examined, however, the cleaved products were more highly expressed in GBM patients as compared to low grade gliomas (Figure 11D).

**DISCUSSION**

Malignant gliomas are a very aggressive type of brain tumors that have a very poor prognosis. Their etiology is unknown. The identification and characterization of a subset of poorly differentiated stem-like cells, called glioma stem cells or BTICs (62, 13, 63), offer an opportunity to understand the genesis of gliomas. Identification of the factors that regulate BTIC proliferation, invasion and tumor development is essential to identify novel therapeutic targets for these aggressive tumors.

By using unbiased serial in vivo selection we identified p75NTR as a novel mediator of glioma (18) and BTIC invasion (19). Expression of p75NTR in human gliomas can make a subset of non-invasive gliomas highly invasive; this effect is enhanced with NGF stimulation. Conversely, the siRNA knockdown of p75NTR reduced the migration of highly invasive glioma cells and BTICs. p75NTR is also implicated in regulating cell survival and proliferation of other types of cancers, in malignant melanoma p75NTR is highly expressed and neurotrophin binding increases melanoma cell invasiveness and promotes survival (64-66), p75NTR activation by NGF promotes survival and proliferation of breast cancer cells (29-31). Whereas, in prostate cancer, bladder cancer and gastric cancer cells p75NTR acts as a tumor suppressor by inducing apoptosis and inhibiting cell proliferation, and invasion (67-70). Taken together these findings suggest that p75NTR expression on some tumor cells enhances their survival and invasion, and in other tumor cells it inhibits survival and invasion. p75NTR’s role in cellular proliferation of human BTICs has not been previously described. In this study, we found that knock down of p75NTR by siRNA decreased BTIC proliferation, binding of NGF to TrkA receptor lead to Trk signaling dependent p75NTR receptor activation by regulated intramembrane proteolysis and this was necessary for BTIC proliferation. NGF and TrkA signaling is known to promote breast cancer cell proliferation, invasion and metastasis (71, 72, 31). We discovered that inhibition of Trk signaling blocked p75NTR cleavage and BTIC proliferation suggesting Trks role in glioma cell proliferation. It is not clear how Trk signaling regulates p75NTR processing.

According to the Cancer Stem Cell hypothesis, glioma stem cells/BTICs are responsible for malignant brain tumor development (62, 73). They express neuronal stem cell markers such as Nestin and Sox2, self-renew, potentially differentiate into many cell type that make up the tumor (10-14, 74, 62, 75) and develop tumors when transplanted into immuno-deficient mice which resemble MGs found in human patients (8, 13, 14). The inhibition of proliferation pathways, such as the Notch (76) and hedgehog pathways (77-79) in glioma stem cells abrogate glioma formation. Similarly we demonstrate here that the inhibition of p75NTR signaling in BTICs inhibits proliferation and, from our previous work, invasion and migration of BTICs (19). These findings indicate that targeting p75NTR in BTICs may be a unique therapeutic strategy for MGs.

Other have reported on the roles of the enzymes ADAM-10 and 17 (which cleave the
ECD of p75NTR and liberate its CTF) in gliomas. The ADAM metalloproteases are required for numerous cellular functions and may be involved in glioma pathogenesis (80, 81). ADAM10, 12 and 17 are implicated in invasion and proliferation of GBM (82-84, 53, 54). TGFβ1 mediates glioma invasion through upregulation of ADAM17/TACE (53). These findings suggest that increased expression and activity of ADAM family members is associated with gliomagenesis. p75NTR is known to be processed by ADAM17/TACE (85, 27) and that p75NTR signaling increases ADAM17 expression which is necessary for apoptotic cell death in sympathetic neurons (27). We found that NGF stimulation of BTICs increases ADAM17 expression (Figure 8F), ADAM10 and ADAM17 levels are increased in malignant glioma (Figure 11A and 11B). Here, we show that the p75NTR-CTF (the cleaved product of ADAM cleavage) is increased in MG patient tumors (Figure 11D). It is also known that ADAMs are required for glioma stem cell sphere formation (86). We found that inhibition of the ADAM metalloproteases blocked the p75NTR proteolysis and prevented glioma invasion in vitro and in vivo (19), and reduced proliferation of BTICs (Figure 6 and 7). ADAM family proteins are required for glioma proliferation and invasion (82-84, 53, 54). Although p75NTR upregulates ADAM17 levels through Jun-kinase activity (27), and ADAM17 enzymatic activity is increased through phosphorylation by the MAP kinase p38 (87), ERK (88) and MEK (46) at Thr735, it is not yet clear which ADAMs are involved in p75NTR processing in malignant glioma and whether they are transcriptionally upregulated or post-translationally modified.

γ-secretase is known to process many membrane receptors (e.g. Notch, Amyloid precursor protein (APP), Erb-4, E-cadherin) and to release ICDs that have signaling capabilities; this has been identified as a novel mechanism in receptor signaling biology (55, 89). It is known that inhibition of γ-secretase blocks glioma stem cells sphere formation, proliferation, survival and increased the differentiation and cell death in vitro and tumor formation in vivo (90-93). It has been speculated that these effects are due to γ-secretase mediated Notch signaling (92, 93). However several studies have now found that p75NTR is also cleaved by the γ-secretase and is required for receptor signaling in neurons where it mediates processes that include inhibition of neurite outgrowth, cell death and cell survival (25, 26, 46-49, 61). Here, we expand the contextual repertoire of p75NTR cleavage by the γ-secretase and show it mediated cellular proliferation in BTICs (Figure 6 and 7).

Here we show that p75NTR activation is also required for proliferation of brain tumor initiating cells in addition to its role in invasion. In our efforts to understand the mechanisms underlie of p75NTR regulated glioma invasion and proliferation, we recently (19) and here demonstrated that the γ-secretase-mediated cleavage of the receptor and release of intracellular domain are required for receptor mediated invasion and proliferation. How receptor cleavage mediates these effects was not known. p75NTR cleavage is necessary for Akt activation and neurotrophin survival in PC12 cells and neurons (50, 46). Further, Akt is implicated in glioma cell proliferation, migration, and invasion, and glioma development and progression (58, 59). Here, we have shown that Akt activation was attenuated in BTICs when receptor proteolysis was blocked (Figure 10).

We previously studied the effect of p75NTR in gliomas (19) largely focused on U87 and U251 human glioma cell lines which do not express endogenous p75NTR (19) and have been in cell culture for decades. Instead we regarded the short-term culture of patient-derived BTICs as more likely to maintain the critical factors and pathways involved in tumor formation. In addition, we did not perform p75NTR knock down in the single BTIC we studied so the effects on proliferation were not apparent. In our current study, we have used four different glioma stem cell lines to understand the role of p75NTR in glioma stem cell proliferation which we examined thoroughly here.

p75NTR signaling is very complex. The receptor interacts with several co-receptors and multiple ligands and activates multiple signaling pathways such as NF-kB and JNK with important physiological consequences (21). The p75NTRs exist as dimers that are linked through conserved cysteine in the transmembrane domain. Upon binding of neurotrophins to p75NTR, dimers...
activate the receptor function via conformational rearrangement, which leads to recruitment of various interactors to the p75NTR ICD and initiation of downstream signaling (94). Several interactors have been identified that can bind to its ICD, including NRIF (95), TRAF6 (42), Rho-GDI (96), NRAGE (97, 98), SC1 (99), RIP2 (41), Bex1 (100) and Fascin (101). There are several examples of the effects of p75NTR cleavage and their interactions with various interactors which are cell type specific. For example, p75NTR undergoes proteolytic cleavage and releases its ICD, which is required for receptor-dependent inhibition of neurite outgrowth through Rho activation (49), induction of apoptosis via TRAF6 dependent ubiquitination and nuclear translocation of NRIF, and activation of JNK (26, 27, 102), cell survival through Akt activation (46) and neoangiogenesis via HIF-1α stabilization (61). Interestingly, Akt (59), JNK (103, 104), and HIF-1α (105, 106) are also required for glioma cell proliferation, migration and invasion. The identity of the proteins which interact with the ICD of p75NTR in MGs to mediate its effects on invasion and proliferation will be an important subject for further study.

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**ABBREVIATIONS USED**

BTIC- Brain Tumor Initiating Cells, GBM- Glioblastoma Multiforme, p75NTR- p75 Neurotrophin Receptor, p75NTR ICD- p75 Neurotrophin Receptor Intracellular Domain, Trk- Tropomyosin Receptor Kinase, NGF- Nerve growth factor, ADAM- a disintegrin and metallopeptidase,

**FIGURE LEGENDS**

**Figure 1: Brain Tumor Initiating Cells express neurotrophin receptors p75NTR, TrkA, TrkB, and TrkC and neurotrophins NGF, BDNF and NT3.**

BTICs (lines 54, 31, G144 and G179) isolated from glioblastoma patient tumors were maintained under proliferative conditions with EGF and FGF, and expression of neurotrophin receptors, expression and secretion of neurotrophic factors were examined. RT-PCR analysis of p75NTR (A), TrkA, TrkB and TrkC (B) were performed using human specific primers, Actin acts as an internal control. Human brain total RNA obtained from Clontech used as positive control (+ve). (C) Quantitative-PCR was performed for TrkA and internal control Actin, expression of TrkA was calculated as ∆∆Ct against the Actin. Data are mean of three independent experiments. Lysates isolated from BTICs were subjected to Western blot analysis (used 60 µg of lysate from each cell line) using antibodies against the p75NTR (D), TrkB (E),
TrkC (F) and Tubulin, FL=Full length receptor, T=Truncated form of receptor. RT-PCR analysis (G) using human specific primers and ELISA (H) using human specific ELISA kits were performed for neurotrophic factors NGF, BDNF and NT3 to determine the expression and secretion, and levels of neurotrophins in the media expressed as ng/ml of media. Data are means ± SD, n=3 independent experiments, (*, p<0.05).

**Figure 2: Knock down of p75NTR decreases proliferation of Brain Tumor Initiating Cells in vitro.**
(A) BTICs (line 54) were transfected with control siRNA (Con-siRNA) or two different p75NTR-siRNAs (p75NTR-siRNA(1) and p75NTR-siRNA(2)) using the Amaxa nucleofector device and Amaxa electroporation kit. After three days in culture, the cells were lysed and subjected to immunoblot analysis with anti-p75NTR antibody. (B) Quantitation of p75NTR Western blots expressed as relative expression level with Actin. Results are means ± SD of three independent experiments, ((*, p<0.05).

BTICs such as line 54 (C), line 31 (D), line G144 (E) and line G179 (F) were transfected with p75NTR-siRNAs or control siRNA (Con-siRNA) using Amaxa electroporation method. 4 days later cells were subjected to MTT assay to assess proliferation. Proliferation is expressed as percent control. n=4 independent experiments (*, p<0.05). BTICs line 54 (G), 31 (H), and G179 (I) were transfected with p75NTR-siRNAs or control siRNA (Con-siRNA), 4 days later cells were subjected to Trypan blue assay to assess cell proliferation. Proliferation is expressed as total number of cells/ml. n=3 independent experiments (*, p<0.05).

**Figure 3: Down regulation of p75NTR decreases Brain Tumor Initiating Cells proliferation in vitro.**
BTICs, lines 54 (A, B), 31 (C), G144 (D) and G179 (E) were transfected with p75NTR-siRNAs or control siRNA (con-siRNA), and after four days in culture, the cells were fixed and processed for immunostaining with the proliferative marker Ki67 and mounted with DAPI containing media. (A) Ki67 (green) and DAPI (blue) staining were imaged by confocal microscopy. (Scale bar, 100 µm). (B) Proliferation was assessed by calculating the number of Ki67 positive cells remaining after four days in culture as a percentage of the total number of DAPI-stained cells and data expressed as percent Ki67 positive cells. n=3 independent experiments (*, p<0.05).

**Figure 4: Neurotrophin NGF stimulates Brain Tumor Initiating Cells proliferation and this is blocked by anti-NGF.**
BTICs (lines 54, 31, G144 and G179) were maintained in neuronal stem cell expansion media and treated with 25, 50 and 100 ng/ml of NGF for three days (NGF was added to cells every 24 hr intervals). The cells were then fixed and immunostained for Ki67 (green) and DAPI (blue), and imaged using confocal microscopy (A) (Scale bar, 100 µm). Then proliferation was assessed by calculating the number of Ki67 positive cells remaining after three days in culture as a percentage of the total number of DAPI stained cells. n=3 independent experiments (B, C, D, E) (*, p<0.05). In some experiments BTICs were treated with NGF antibody or control-IgG (Con-IgG) (F-I) for 4 days and then assessed the proliferation as described above (*, p<0.05).

**Figure 5: Neurotrophins BDNF and NT3 stimulate Brain Tumor Initiating Cells proliferation.**
BTICs 54, 31, G144 and G179 were maintained in neuronal stem cell expansion media with growth factors, and treated with neurotrophin BDNF (50 ng/ml) for three days (BDNF was added to cells every 24 hr intervals). The cells were then fixed and immunostained for proliferative marker Ki67 (green) and DAPI (blue), and imaged using confocal microscopy (A) (Scale bar, 100 µm). Proliferation rate for all lines (C) were assessed by calculating the number of Ki67 positive cells remaining after three days in culture as a percentage of the total number of DAPI stained cells. Data expressed as percent Ki67 positive cells. n=3 independent experiments (*, p<0.05).

BTICs 54, 31, G144 and G179 were maintained in neuronal stem cell expansion media with growth factors EGF and FGF and treated with 50 ng/ml of NT3 for three days (NT3) was added to cells every 24 hr intervals). Three days later cells were fixed and immunostained for Ki67, a proliferative marker (green)
and DAPI (blue), and imaged using microscopy (B) (Scale bar, 100 µm). The proliferation was assessed by calculating the number of Ki67 positive cells as a percentage of the total number of DAPI stained cells and expressed as percent Ki67 positive cells (D). n=3 independent experiments. (*, p<0.05).

**Figure 6: NGF stimulates p75NTR cleavage, inhibition of cleavage by α- and γ-secretase inhibitors blocks NGF mediated receptor proteolysis and proliferation of Brain Tumor Initiating Cells.**

(A) BTICs (line 54) were maintained in neurobasal media with EGF and FGF for 2 days, then cells were washed twice with fresh media and treated with 1µM epoxomycin (proteosome inhibitor) alone or together with 100 ng/ml of NGF in the presence or absence of γ-secretase inhibitor DAPT (200 nM) or metalloprotease inhibitor TAPI-2 (500 nM) for 6 hrs. Then cells were lysed and performed Western blot analysis using antibodies against the p75NTR-ICD and Tubulin. U251 expressing p75NTR treated with epoxomycin used as positive control. FL=full-length p75NTR; CTF=p75NTR C-terminal fragment; ICD=p75NTR intracellular domain. Data are representative of three independent experiments.

Cell line 54 (B, C), 31 (D, E), G144 (F, G), G179 (H, I) were cultured in neurobasal media 12 hr, and then treated the cells with NGF alone or along with γ-secretase inhibitor DAPT (200 nM) (B, D, F, H) or metalloprotease inhibitor TAPI-2 (500 nM) (C, E, G, I) or left untreated for three days. Cells were added with NGF, DAPT and TAPI-2 for every 24 hr. Three days later, cells were fixed, stained for Ki67 and DAPI, and Ki67 positive cells were quantified. Data shown are means of ±SD, n=3 independent experiments (*, p<0.05). ** indicates significant difference with control with p<0.05.

**Figure 7: Expression of γ-secretase resistant mutant p75NTR blocks proliferation of Brain Tumor Initiating Cells.**

(A) BTICs (line 54) were transfected with wildtype-p75NTR or γ-secretase resistant mutant p75NTR (p75FasTM) using the Amaxa nucleofector device and Amaxa electroporation kit or left untransfected. After three days the cells were washed twice with fresh media and treated with 100 ng/ml of NGF and 1μM epoxomycin for 6 hr, and then cells were lysed and subjected to Western blot analysis with p75NTR-ICD antibody and Tubulin. FL=full-length p75NTR; CTF=p75NTR C-terminal fragment; ICD=p75NTR intracellular domain. Data are representative of 3 independent experiments.

BTICs such as 54 (B), 31 (C), G144 (D) and G179 (E) were transfected as above with wild type-p75NTR or p75FasTM or left untransfected. Two days after transfection, the cells were treated with 100ng/ml of NGF for three days (NGF was added to media every 24 hr). Three days after NGF treatment cells were fixed, stained for Ki67 and DAPI, and Ki67 positive cells were quantified and data was expressed as percent Ki67 positive cells. Data shown are means of ±SD, n=3 independent experiments (*, p<0.05).

**Figure 8: Inhibition of Trk signaling attenuates NGF stimulated Brain Tumor Initiating Cells proliferation and p75NTR cleavage.**

BTIC line 54 (A), 31 (B), G144 (C), G179 (D) were split and maintained in neurobasal media for 12 hr, and then treated the cells with 50 ng/ml of NGF alone or along with Trk inhibitor K252a (200 nM) or left untreated for three days. Cells were added with NGF and K252a for every 24 hr. Three days later, cells were fixed, stained for Ki67 and DAPI, and Ki67 positive cells were quantified. Data shown are means of ±SD, n=3 independent experiments (*, p<0.05).

(E) BTIC line 54 were maintained in neurobasal media for 2 days, then washed with fresh media and treated with 50 ng/ml of NGF in the presence or absence of Trk inhibitor K252a (200 nM) and with 1µM epoxomycin for 6 hrs. Then cells were lysed and subjected to Western blotting using p75NTR-ICD and Tubulin antibodies. FL=full-length p75NTR; CTF=p75NTR C-terminal fragment; ICD=p75NTR intracellular domain. Data are representative of three independent experiments.

(F) BTIC line 54 were maintained in neuronal stem cell expansion media containing EGF and FGF, and treated with 50ng/ml of NGF for 2 and 6 hr. Then total RNA was isolated, reverse-transcribed and performed quantitative-PCR for ADAM17/TACE and Actin, expression of ADAM17 was calculated as ΔΔCt against the Actin. Data are mean of two independent experiments.
Figure 9: p75NTR-ICD expression induces proliferation of Brain Tumor Initiating cells.
BTIC line 54 were transfected with GFP alone or co-transfected with GFP and p75NTR intracellular
domain construct (p75ICD) using Amaza electroporation method and cells were maintained in media
with growth factors. 2 days later cells were lysed and performed p75ICD and Tubulin Western blotting
analysis (A). Three days after transfection, cells were fixed, stained for Ki67 (red) and imaged using
microscopy (B) (Scale bar, 50 µm). The proliferation was scored by calculating the number of Ki67
positive cells in GFP positive cells (C). Data shown are means of ±SD, n=3 independent experiments (*,
p<0.05).

Figure 10: p75NTR cleavage is required for Akt activation in Brain Tumor Initiating cells.
(A) BTIC line 54 were electroporated using Amaza electroporation method with control-siRNA
(Control), p75NTR-siRNAs or γ-secretase mediated resistant mutant p75NTR (p75FasTM) and cells were
maintained in neurobasal media without growth factors for 2 days. Then cells were added with growth
factors EGF and FGF for 6 hr, cells were lysed and performed Western blotting for p75NTR, phospho-
Akt and Actin. (B) p-Akt and Actin Western blots were quantified and expressed as relative p-Akt/Actin
ratio. N=3 independent experiments (*, p<0.05).

Figure 11: p75NTR and signaling components associated with receptor proteolysis, and p75NTRs
cleaved products, carboxy terminal fragment (CTF) and intracellular domain (ICD) are elevated in
malignant glioma patient tumor specimens.
Microarray data set of 528 GBM patients and 10 normal tissue from TCGA (A) and Moffitt Cancer
Center data set of 108 GBM patients (B) for expression analysis p75NTR, its co-receptor TrkA/NTRK1,
ligand NGF, α-secretases (ADAM10 and ADAM17) and γ-secretase components such as presenelin1 and
2 (PSEN1 and PSEN2), Nicastrin (NCSTN), and APH1. We considered that values above 2 of log2
expression value are significantly higher. (C) Frozen tumor specimen from grade I to grade IV of
malignant gliomas obtained from Moffitt Cancer Center, isolated total-RNA, made cDNA and performed
RT-PCR analysis for p75NTR, NGF and Actin analysis using human specific primers. Human brain total
RNA obtained from Clontech served as positive control (+ve). (D) Frozen tumor specimen were lysed
and subjected to Western blotting analysis of p75NTR using p75NTR-ICD antibody which recognizes
full length (FL), carboxy terminal fragment (CTF) and intracellular domain (ICD) of the receptor. Tubulin
was used as loading control and U251 cells expressing p75NTR used as positive control for p75NTR.
JPA (1) =Juvenile Pilocytic Astrocytoma, GA= Gemistocytic Astrocytoma, LGA= Low Grade
Astrocytoma, LGO= Low Grade Oligodendroglioma, AOA= Anaplastic Oligoastrocytoma, AA=
Anaplastic Astrocytoma, GBM= Glioblastoma Multiforme. RT & Chemo indicates patient tumor treated
with radiation and Temozolomide (chemotherapy) before surgery.
Figure 1

A

B

C

D

E

F

G

H

p75NTR Regulates BTIC Proliferation
Figure 2

A

B

C

D

E

F

G

H

I

p75NTR Regulates BTIC Proliferation

p75NTR-WB

Tubulin-WB

Relative p75NTR intensity/Tubulin

Proliferation (% Control)

Proliferation (% Control)

Proliferation (% Control)

Proliferation (% Control)

Total number of cells/ml

Total number of cells/ml

Total number of cells/ml

54

31

G144

G179

54

31

G179
Figure 3

A

| DAPI | Ki67 | Overlay |
|------|------|---------|
| Control-siRNA | ![Control-siRNA DAPI](image) | ![Control-siRNA Ki67](image) | ![Control-siRNA Overlay](image) |
| p75NTR-siRNA(1) | ![p75NTR-siRNA(1) DAPI](image) | ![p75NTR-siRNA(1) Ki67](image) | ![p75NTR-siRNA(1) Overlay](image) |
| p75NTR-siRNA(2) | ![p75NTR-siRNA(2) DAPI](image) | ![p75NTR-siRNA(2) Ki67](image) | ![p75NTR-siRNA(2) Overlay](image) |

B

% Ki67 Positive Cells

|          | Con-siRNA | p75NTR-siRNA(1) | p75NTR-siRNA(2) |
|----------|-----------|-----------------|-----------------|
| #54      | 70        | 30              | 40              |

C

% Ki67 Positive Cells

|          | Con-siRNA | p75NTR-siRNA(1) | p75NTR-siRNA(2) |
|----------|-----------|-----------------|-----------------|
| #31      | 70        | 50              | 60              |

D

% Ki67 Positive Cells

|          | Con-siRNA | p75NTR-siRNA(1) | p75NTR-siRNA(2) |
|----------|-----------|-----------------|-----------------|
| G144     | 70        | 50              | 60              |

E

% Ki67 Positive Cells

|          | Con-siRNA | p75NTR-siRNA(1) | p75NTR-siRNA(2) |
|----------|-----------|-----------------|-----------------|
| G179     | 70        | 50              | 60              |
Figure 4

A  
DAPI  Ki67  Overlay  
Control  NGF  

B  
54  
% Ki67 Positive Cells  
Con  NGF (25ng)  NGF (50ng)  Con  NGF (100ng) 

C  
31  
% Ki67 Positive Cells  
Con  NGF (25ng)  NGF (50ng)  Con  NGF (100ng) 

D  
G144  
% Ki67 Positive Cells  
Con  NGF (25ng)  NGF (50ng)  Con  NGF (100ng) 

E  
G179  
% Ki67 Positive Cells  
Con  NGF (25ng)  NGF (50ng)  Con  NGF (100ng) 

F  
54  
% Ki67 Positive Cells  
Con  NGF Antibody  
Con -IgG  

G  
31  
% Ki67 Positive Cells  
Con  NGF Antibody  

H  
G144  
% Ki67 Positive Cells  
Con  NGF Antibody  

I  
G179  
% Ki67 Positive Cells  
Con  NGF Antibody
Figure 5

A

B

C

D

p75NTR Regulates BTIC Proliferation
Figure 6

A

B

C

D

E

F

G

H

I

p75NTR Regulates BTIC Proliferation
Figure 7

A

|          | Un transfected | WT-p75NTR | p75FasTM |
|----------|----------------|-----------|----------|
| NGF      | -              | +         | -        |
| FL       |                |           |          |
| p75NTR-WB|                |           |          |
| CTF      |                |           |          |
| ICD      |                |           |          |
| Tubulin-WB|              |           |          |

B

\[
\% \text{ Ki67 Positive Cells} \]

Con | NGF Un transfected | NGF WT-p75NTR | NGF p75FasTM | \(*\) |
-----|-------------------|--------------|--------------|------|
0     | 20                | 50           | 50           | 80   |
50    | 50                | 50           | 50           | 50   |
80    | 50                | 50           | 50           | 50   |

C

\[
\% \text{ Ki67 Positive Cells} \]

Con | NGF Un transfected | NGF WT-p75NTR | NGF p75FasTM | \(*\) |
-----|-------------------|--------------|--------------|------|
0     | 20                | 50           | 50           | 80   |
50    | 50                | 50           | 50           | 50   |
80    | 50                | 50           | 50           | 50   |

D

\[
\% \text{ Ki67 Positive Cells} \]

Con | NGF Un transfected | NGF WT-p75NTR | NGF p75FasTM | \(*\) |
-----|-------------------|--------------|--------------|------|
0     | 20                | 50           | 50           | 80   |
50    | 50                | 50           | 50           | 50   |
80    | 50                | 50           | 50           | 50   |

E

\[
\% \text{ Ki67 Positive Cells} \]

Con | NGF Un transfected | NGF WT-p75NTR | NGF p75FasTM | \(*\) |
-----|-------------------|--------------|--------------|------|
0     | 20                | 50           | 50           | 80   |
50    | 50                | 50           | 50           | 50   |
80    | 50                | 50           | 50           | 50   |
Figure 9

A

B

GFP

Ki67

Overlay

C

p75NTR Regulates BTIC Proliferation

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Figure 10

A

Control  p75NTR-siRNA  p75fasTM

B

p75NTR-WR

p-Akt-WR

Actin-WB

p-Akt/Actin

Con  p75NTR-siRNA  p75fasTM

*
p75 Neurotrophin Receptor Cleavage by α- and γ-Secretases is required for Neurotrophin mediated proliferation of Brain Tumor Initiating Cells.
Peter A. Forsyth, Niveditha Krishna, Samuel Lawn, J. Gerardo Valadez, Xiaotao Qu, David A. Fenstermacher, Michelle Fournier, Lisa Potthast, Prakash Chinnaiyan, Geoffrey T. Gibney, Michele Zeinieh, Philip A. Barker, Bruce D. Carter, Michael K. Cooper and Rajappa S. Kenchappa

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