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Cell Autonomous and Cell Non-Autonomous Roles of p75 Neurotrophin Receptor (p75NTR) in Glioblastoma Progression

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Cell Autonomous and Cell Non-Autonomous Roles of p75 Neurotrophin Receptor (p75NTR) in Glioblastoma Progression

by

Mana Alshehri

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN MEDICAL SCIENCE

CALGARY, ALBERTA

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Abstract

Glioblastoma multiforme (GBM) is the most common and aggressive brain tumor that is inevitably a fatal disease. GBM is a heterogeneous tumor consisting of tumor cells and a small population known as brain tumor initiating cells (BTICs) or glioblastoma stem-like cells. BTICs appear to drive tumor progression, underlie therapeutic resistance to current treatment and tumor relapse and have been highlighted as important therapeutic targets. The ability of glioma cells to invade into the surrounding brain parenchyma is a major clinical issue rendering glioblastoma incurable by conventional therapies. Using a large panel of GBM cells including genetically different patient-derived-BTICs we have investigated the cell autonomous and cell non-autonomous roles of p75NTR in GBM progression. Immunohistochemical studies and western blot analyses demonstrated that p75NTR is variably expressed on BTICs. Loss-of-function and gain-of-function studies of p75NTR revealed that p75NTR is involved in regulating self-renewal, proliferation, cell cycle progression, symmetry and asymmetry cell divisions, apoptosis, differentiation and invasion of genetically different patient-derived BTICs. Furthermore, loss-of-function of p75NTR in these BTICs inhibited their tumorigenic behaviors in vivo and extended the survival time of mice bearing brain tumors generated by p75NTR knockdown BTICs compared to their control counterparts. In addition to the cell autonomous roles of p75NTR in regulating GBM progression, this thesis provided novel findings about the cell non-autonomous roles of p75NTR in mediating glioma invasion. We further demonstrated that p75NTR is transferred between glioma cells through extracellular vesicles (EVs) to induce invasive properties in recipient glioma cells. Interestingly, proteolytic processing of p75NTR was detected in EVs and was required for meditating glioma cell invasion by p75NTR-containing EVs, as EVs isolated from glioma cells expressing a cleavage-resistant chimera of p75NTR (p75Fas-S) or from cells treated with a γ-
secretase inhibitor failed to induce glioma cell invasion. $p^{\text{NTR}}_7$ was also found in EVs isolated from sera of mice bearing brain tumors generated by $p^{\text{NTR}}_7$-expressing BTICs. These data highlight a previously unknown function of this receptor and suggest it may be a novel therapeutic target in the treatment of this devastating cancer.
Preface

During the period of my PhD degree, I contributed in the following scientific papers:

**Published papers/ book chapter**


**Manuscripts under preparation**

**p75 neurotrophin receptor (p75\textsuperscript{NTR}) regulates self-renewal, proliferation and differentiation of brain tumour initiating cells**

(leading author, Final stage of preparation)

**Intracellular transfers of p75 neurotrophin receptor (p75\textsuperscript{NTR}) by extracellular vesicles derived from glioma cell promote glioma invasion.**

(Leading author, Final stage of preparation)

**CD271 (p75 Neurotrophin receptor) signalling drives metabolic plasticity in glioma.**

(co-author, Final stage of preparation)

**Glioma-derived IL33 orchestrates the brain tumor microenvironment to promote glioma progression**

(Co-author: Final stage of preparation)

**Capicua regulates neural progenitor cell proliferation and oligodendrocyte differentiation**

(Co-author: Final stage of preparation)
Acknowledgements

This thesis could not have been completed without the assistance and contributions of many people. Thank you to Dr. Steve Robbins, who provided me with the opportunity to work in his lab and was a source of constant encouragement and support. Steve’s innovative ideas and thoughtful interpretations about diverse scientific questions has helped me to learn and acquire divergent thinking. I want to express my deepest appreciation to Dr. Donna Senger, for her guidance, support, advice and constant encouragement throughout the course of this thesis.

Thank you also to my committee members, Dr. Gregory Cairncross, Dr. Jennifer Chan and Dr. Carol Schuurmans who were always available for advice and support when needed.

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<tr>
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<th>Abbreviation and Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>ADAMs</td>
<td>a disintegrin and metalloproteinase,</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<tr>
<td>ARRD1</td>
<td>arrestin domain-containing protein 1</td>
</tr>
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<td>BBB</td>
<td>blood brain barrier</td>
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<td>BCA</td>
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<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<td>brain tumor initiating cells</td>
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<td>bromodeoxyuridine</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>Cdh-11</td>
<td>cadherin-11</td>
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<tr>
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<td>cancer stem cells</td>
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<td>cDNA</td>
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<tr>
<td>CIC</td>
<td>capicua transcriptional repressor</td>
</tr>
<tr>
<td>CLIC1</td>
<td>chloride intracellular channel-1</td>
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<td>central nervous system</td>
</tr>
<tr>
<td>CRD</td>
<td>cysteine-rich domain</td>
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<td>C-terminal fragment</td>
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<td>CDKN2A</td>
<td>cyclin dependent kinase inhibitor 2A</td>
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<td>conditioned medium</td>
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<td>cytomegalovirus</td>
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<td>cerebrospinal fluid</td>
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<td>3,3’-diaminobenzidine tetrahydrochloride</td>
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<td>DAPI</td>
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<td>death domain</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<td>DNA</td>
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<td>DRG</td>
<td>dorsal root ganglia</td>
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<td>ECD</td>
<td>extracellular domain</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGFRvIII</td>
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<tr>
<td>ESCRT</td>
<td>endosomal sorting complex required for transport</td>
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<tr>
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<td>Eph</td>
<td>ephrin</td>
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<td>extracellular vesicles</td>
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<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FUBP1</td>
<td>far upstream element binding protein 1</td>
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<tr>
<td>GAGs</td>
<td>glycosaminoglycans</td>
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GAPDH  glyceraldehyde-3-phosphate dehydrogenase
GBM    glioblastoma
G-CSF  granulocyte colony-stimulating factor
GDP    guanosine 5’-diphosphate
GFAP   glial fibrillary acidic protein
GFP    green fluorescent protein
GTP    guanosine 5’-triphosphate
HA     hyaluronic acid
HEK    human embryonic kidney
HEPES  N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)
HGF    hepatocyte growth factor
Hh     Hedgehog
HRP    horseradish peroxidase
HSPG   heparan sulfate proteoglycan
Iba-1  ionized calcium-binding adapter molecule 1
ICD    intracellular domain
IDH1   isocitrate dehydrogenase 1
IDH2   isocitrate dehydrogenase 2
IL     interleukin
ILVs   intraluminal vesicles
IPA    Ingenuity Pathway Analysis
JM     juxtamembrane region
JNK    c-jun N-terminal kinase
kb     kilobase
kDa    kilodaltons
LINGO  LRR and Ig domain-containing Nogo receptor interacting protein
LPS    lipopolysaccharide
MAG    myelin-associated glycoprotein
MGMT   methylguanine methyltransferase
MMR    functional mismatch repair
MMP    matrix metalloproteinase
MRI    Magnetic resonance imaging
mRNA   messenger RNA
MV    microvesicles
MVBs   multi-vesicular bodies
NAD    p75 neurotrophin receptor-associated cell death executor
NCSCs  neural crest stem cells
NF1    neurofibromatosis type 1
NCAM   neural cell adhesion molecule
NF-κB  nuclear factor-κB
NGF    nerve growth factor
NgR    Nogo receptor
NRAGE  neurotrophin receptor-interacting MAGE homologue
NRIF   neurotrophin receptor interacting factor
NT     neurotrophin
OMgp   oligodendrocyte-myelin glycoprotein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>p75&lt;sup&gt;NT&lt;/sup&gt;R</td>
<td>p75 neurotrophin receptor</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>PARP</td>
<td>poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PB</td>
<td>PiggyBac</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC12</td>
<td>pheochromocytoma cell line</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
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<tr>
<td>p75Fas-S</td>
<td>cleavage-resistant chimera of p75&lt;sup&gt;NT&lt;/sup&gt;R</td>
</tr>
<tr>
<td>RIP</td>
<td>regulated intramembrane proteolysis</td>
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<td>platelet-derived growth factor receptor A</td>
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<tr>
<td>siRNA</td>
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<td>SM</td>
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<td>serum-free culture medium</td>
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<td>SVZ</td>
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<td>Tris buffered saline containing 0.2 % Tween-20</td>
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<td>telomerase reverse transcriptase</td>
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<td>TNF</td>
<td>tumour necrosis factor</td>
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<td>tumour necrosis factor receptor</td>
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<td>TP53</td>
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<td>TNF receptor-associated death domain</td>
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<td>TRAF</td>
<td>TNF receptor-associated factor</td>
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<td>TRAIL</td>
<td>TNF-Related Apoptosis Inducing Ligand</td>
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<td>Transmission electron microscopy</td>
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<td>Trk</td>
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<td>TSG101</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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Chapter one: Introduction

1.1 Diffuse Glioma

1.1.1 Classification of Diffuse gliomas

Diffuse glioma is a diverse set of the most common primary brain tumors, especially in adult patients. Historically, classification of glioma was based on histopathologic appearance, delineating tumors as diffuse astrocytomas (with glioblastoma as its most frequent and most malignant representative), oligodendroglioma, and mixed oligoastrocytoma, tumors with a mixed astrocytic and oligodendroglial phenotypes. In 2007, The World Health Organization (WHO) used histopathological grading criteria to classify glioma into four grades of increasing severity (WHO grade I–IV) with astrocytic tumors representing around 82% of the grade IV tumors. The remaining 18% consists of unspecified glioma (7%), oligodendroglioma (6%), and mixed oligoastrocytoma (3%), with the latter categories also classified into WHO grade II–III (D. N. Louis et al., 2007).

Grade I gliomas are non-invasive, well circumscribed tumors, have low proliferative ability and are usually cured following surgical resection alone. Grade II gliomas, although of low proliferative potential, are infiltrative tumors that tend to recur after surgical resection and can develop into higher grades. Grade III gliomas are aggressive anaplastic tumors characterized by nuclear atypia, dense cellularity, and elevated mitotic activity, and often progress to grade IV gliomas. Grade IV glioma, known as glioblastoma multiforme (GBM), are either (i) primary GBMs that arise rapidly de novo, without clinical or histologic evidence of a less malignant precursor lesion and represent around (~90%) of grade IV cases, or (ii) secondary glioblastomas that progress from low-grade diffuse astrocytoma or anaplastic astrocytoma. In addition to nuclear
atypia, dense cellularity, and elevated mitotic activity, these tumors are also characterized by microvascular proliferation and necrosis (D. N. Louis et al., 2007).

1.1.2 The Updated Classification of diffuse gliomas

In the past decade, knowledge of molecular alterations in gliomas has massively expanded. Previously, molecular information was mainly provided as supplementary information within histologically defined grades, and tumors were categorized based on their histology. More recently, molecular studies have revealed diverse genetic alterations in the different glioma grades. Thus, the new 2016 WHO glioma classification has not only integrated molecular information, but in some cases molecular classifications override the histological diagnosis (D. N. Louis et al., 2016). All human gliomas previously categorized based on histologic grades have now been subdivided into three main groups based on two parameters: the mutational status of isocitrate dehydrogenase 1 (IDH1), or its mitochondrial cousin, isocitrate dehydrogenase 2 (IDH2) and the co-deletional status of chromosomal arms 1p and 19q. The groups are: (1) astrocytoma with IDH–wild type; (2) astrocytoma with IDH-mutant and (3) oligodendroglioma with both IDH-mutant and 1p/19q-co-deletetion (D. N. Louis et al., 2016).

Each group of diffuse glioma is further associated with recurrent somatic alterations in other loci. For instance, astrocytomas harboring the IDH–wild-type, including glioblastoma (GBM) wild type IDH, are frequently associated with amplifications in epidermal growth factor receptor (EGFR), loss of genetic material on chromosome 10, including the loss of phosphatase and tensin homolog (PTEN), and deletions of cyclin dependent kinase inhibitor 2A/B (CDKN2A/B) (Cancer Genome Atlas Research, 2008; Network, 2013; Parsons et al., 2008). In addition, astrocytomas harboring IDH mutations are also frequently mutated for tumor protein p53 (TP53) and ATRX, whereas oligodendrogliomas are frequently associated with mutations in FUBP1 or
CIC (Eckel-Passow et al., 2015). Moreover, the presence of the mutated telomerase reverse transcriptase (TERT) promoter is inversely correlated with the mutated ATRX, an alteration characteristic of both IDH–wild-type infiltrating astrocytomas and oligodendrogliomas (Cancer Genome Atlas Research et al., 2015; Eckel-Passow et al., 2015; Nonoguchi et al., 2013). Figure 1.1 summarizes the WHO 2016 classification of diffuse gliomas.
Figure 1.1: 2016 WHO classification of diffuse glioma tumors

- **2016 WHO of diffuse gliomas**
  - **Diffuse Glioma**
    - **IDH-status**
      - **IDH-mutant**
        - Genetic alterations: 1p/19q co-deletion, TERT
      - **IDH-wild type**
        - Genetic alterations: 1p/19q intact, TP53, ATRX, EGFR, PTEN, CKDN2A
    - **Classification**
      - Oligodendroglioma
      - Glioblastoma (Secondary)
      - Glioblastoma (primary)
    - **Prognosis**
      - Good prognosis
      - Intermediate prognosis
      - Poor prognosis
1.1.3 Glioblastoma multiforme

Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor, characterized by high vascular endothelial proliferation, necrosis, cellular atypia, increased mitotic activity, and high cellular density. Despite current therapies including surgical tumor resection followed by radiotherapy and chemotherapy with temozolomide (TMZ), an oral alkylating agent which can cross the blood brain barrier (BBB), GBM patients’ prognosis remains disappointing and survival is limited on average to 14.6 months with less than 5% of patients surviving up to 5 years (Stupp et al., 2005). GBMs that harbor the wild-type IDH are classically termed “de novo glioblastoma” or “primary glioblastoma” that develop quickly, have no clinically evident lower-grade precursor, and are molecularly distinct from secondary GBMs which are currently classified as IDH mutant GBMs.

GBM tumors are characterized by a marked heterogeneity at both the cellular and molecular levels, from which the term ‘multiforme’ originated. Early findings of The Cancer Genome Atlas Research Network (TCGA) revealed recurrently altered core pathways involving receptor tyrosine kinases (RTKs), TP53, and retinoblastoma protein (RB) (Cancer Genome Atlas Research, 2008; Cancer Genome Atlas Research et al., 2015). In addition, global gene expression analysis revealed that there are several molecular subtypes of adult GBM. These subtypes were classified as “proneural,” “neural,” “mesenchymal,” and “classical” (Verhaak et al., 2010).

Classical GBM tumors are associated with abnormally high levels of EGFR and the presence of the mutated EGFR variant III (EGFRvIII), a gain-of-function mutation that arises from genomic deletion of exons 2–7, results in a ligand-independent, constitutively active receptor. Clinically, the classical group survives the longest of all subtypes in response to aggressive treatment (Verhaak et al., 2010).
Unlike the classical subtype, the proneural subtype typically carries TP53 mutations (54 percent) and also is characterized by mutations mainly in IDH1, with a few cases in IDH2, as well as amplification and mutations in platelet-derived growth factor receptor A (PDGFRA). Clinically, proneural tumors are found in younger patients who tend to survive longer, however, patients with proneural tumors who received aggressive treatment do not survive significantly longer than proneural patients who do not receive aggressive treatment (Verhaak et al., 2010).

The mesenchymal subtype is associated with mutated NF1 (37 percent) as well as mutations in the tumor suppressor genes PTEN and TP53. Although mesenchymal patients have poor survival compared to other groups, patients in the mesenchymal group have significant increases in survival after aggressive treatment (Verhaak et al., 2010).

The neural subtype is characterized by the expression of several genes that are also typical of the normal brain, mainly non-cancerous nerve cells, or neurons. Clinically, patients in the neural subtype are the oldest and show some improvement in survival after aggressive treatment, but not to the extent as the classical and mesenchymal groups (Verhaak et al., 2010).

Subsequent work on a set of GBMs obtained from all age groups used global DNA methylation patterns to identify six distinct GBM subgroups including “IDH,” “K27,” “G34,” “RTK I (PDGFRA),” “Mesenchymal,” and “RTK II (Classic)”. The clusters, “K27” and “G34,” were enriched in pediatric and young adult patients and corresponded to tumors with mutations in histone genes such as H3F3A (Sturm et al., 2012). Based on the results of this study, GBM can first be classified into two major categories based on IDH status: GBM IDH-wild type or GBM with mutated IDH. In the GBM IDH-wild type group, there are several molecular alterations that are excluded from IDH-mutant GBMs, including EGFR amplification seen in the “classical” or “RTK-II” subvarieties (Sturm et al., 2012). Collectively, all these findings revealed that GBM
tumors are extensively heterogeneous and harbor diverse genetic alterations, suggesting that not all GBM patients can benefit from the same treatment and that personalized medicine could be more effective in treating GBM patients.

1.1.4 Glioblastoma heterogeneity

Although the classification of GBMs into several distinct molecular subtypes revealed the genetic heterogeneity between GBM tumors (Cancer Genome Atlas Research, 2008; Cancer Genome Atlas Research et al., 2015; Verhaak et al., 2010), recent studies showed that these GBM subtypes are flexible, have a high degree of plasticity and vary spatially and temporally within the same tumor (Patel et al., 2014; Reinartz et al., 2017; Szerlip et al., 2012). Single cell RNA-sequencing showed that an individual GBM harbours a heterogeneous mixture of cells representing all the distinct GBM subtypes. Furthermore, analysis of the proneural subtype tumors, which were previously associated with the best survival time among other subtypes, showed that these tumors also have markers of other subtypes and correlated with poor survival, particularly if the relative representation of the alternative subtypes was high in the tumor (Patel et al., 2014). The intratumor heterogeneity has also been found to correlate with therapeutic resistance in GBM tumors, as single cell derived GBM sub-clones showed distinct genetic identities and differential drug resistance profiles (Reinartz et al., 2017). In addition, sampling different regions of GBMs revealed that these tumors harbour spatial and temporal heterogeneity and suggested that designating a GBM to a particular subgroup based on previous genomic methodology and molecular subtyping was performed from single regional biopsies, and thus were not representative of an entire tumor. Thus, distinct samplings of a single tumor may be classified into different GBM subtypes (Sottoriva et al., 2013). These findings suggest that in addition to the existence of extensive genomic heterogeneity in GBM tumors, there is also clonal heterogeneity that
contributes to resistance to chemotherapy and radiotherapy, leading to tumor recurrence. In line with this notion, genomic mutational analysis of primary GBMs and their paired recurrences showed that these tumors were highly divergent and only shared a small number of early mutations (Johnson et al., 2014). In 43% of analysed GBM cases, at least half of the genetic alterations in the primary tumors were undetectable at recurrence (Johnson et al., 2014), suggesting that chemotherapy and radiotherapy generates a selective environment for minor clones existing at the time of initial diagnosis.

A further layer of complexity in GBM arose from the cellular heterogeneity present in the glioma microenvironment, which is comprised of neoplastic and non-neoplastic cells including the vasculature, the various infiltrating and resident immune cells and other glial cell types. Functional GBM heterogeneity arises from existence of various cellular subpopulations of neoplastic cells that have acquired stem cell properties of self-renewal and multi-lineage differentiation, variably labeled in the literature as glioma stem cells (GSCs) or brain tumor initiating cells (BTICs). These cells and the infiltrative cells that migrate away from the GBM tumor bulk are likely to act as disease reservoirs involved in tumor progression, therapy resistance and tumor relapse.

1.1.5 Glioblastoma Disease Reservoirs

1.1.5.1 Brain Tumor Initiating Cells (BTICs)

Previously, it was thought that neoplastic tumor cells are relatively homogenous and all of the tumor cells have the ability to produce more cancer cells, therefore, the goal of cancer treatment has been to target all tumor cells. This theory has been challenged by the cancer stem cell (CSC) hypothesis; that a small population of tumor cells with stem-like properties is the main candidate for tumor growth, chemo- and radiotherapy resistance, and tumor recurrence. Many recent studies have focused on brain tumor stem cells or cells that initiate tumorigenesis (brain tumor-initiating
cells; BTICs or sometime called glioma stem cells (GSC, for this thesis we will adhere to the BTIC nomenclature) (Bao et al., 2006; Hemmati et al., 2003; S. K. Singh et al., 2004). The existence of these cells was first postulated based on the observation that many cancers are organized as a hierarchy that consists of different classes of cells with tumorigenic and differentiation potential. The presence of these stem-like cells has now been identified in several tumor types, including malignant glioma (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003; Bonnet & Dick, 1997; Chiba et al., 2007; Collins, Berry, Hyde, Stower, & Maitland, 2005; Eramo et al., 2008; O'Brien, Pollett, Gallinger, & Dick, 2007; Ricci-Vitiani et al., 2007; S. K. Singh et al., 2004) where these cells have been shown to maintain the ability to self-renew, proliferate, and give rise to more differentiated daughter cells that can repopulate the complexity of the tumor (Bao et al., 2006; Hemmati et al., 2003; S. K. Singh et al., 2003; S. K. Singh et al., 2004). Although there is great debate in the field as to the validity and nature of a bona fide cancer stem cell population (Rowan, 2009), their importance remains. These cells have signature characteristics of transformed cells including growth factor independence and tumorigenicity, they harbor the spectrum of molecular genetic alterations that are known to occur in human glioblastoma [e.g., mutations of the TP53, PTEN, IDH1, EGFR (Blough, Beauchamp, Westgate, Kelly, & Cairncross, 2011; Kelly et al., 2009; Luchman et al., 2012; Lun et al., 2016)], and they form tumors that look and behave like human glioblastoma; infiltrating the cerebral cortex, spreading across the subependyma, and migrating across the corpus callosum (Kelly et al., 2009; S. K. Singh et al., 2004).

Although CD133 (prominin-1) is the most commonly used cell surface marker to identify BTICs (S. K. Singh et al., 2004), other markers including CD15 (also called Lewis x and SSEA-1 [stage-specific embryonic antigen 1]) (Son, Woolard, Nam, Lee, & Fine, 2009), CD44 (Liu et al., 2006), Integrin α6 (Lathia et al., 2010), SOX2 (Hemmati et al., 2003) and olig2 (Ligon et al., 2007)
have been identified. Despite the importance of all these markers, which are expressed by both neural stem cells and BTICs, it is unlikely that a single marker will ever be uniformly informative for BTICs because GBM tumors probably contain multiple populations of BTICs expressing different markers. Therefore, the definition of BTICs remains functional, requiring both sustained self-renewal ability and tumor propagation (J. Chen, McKay, & Parada, 2012).

1.1.5.2 The invasive glioma disease reservoir

Infiltration of glioma cells throughout the brain is a hallmark of GBM, making complete surgical resection impossible. In approximately 95% of cases, recurrent GBM grow within a few centimeters of the resection cavity. This invasive behavior of glioma cells is mediated by distinct molecular processes including detachment of glioma cells from the main tumor mass, interaction of the cell with the surrounding extracellular matrix (ECM), degradation of the ECM to provide passage for the cell, and activation of cellular machinery underlying cell movement (Furnari et al., 2007; Nakada et al., 2007). As mentioned, this invasive process has been reported to occur within the host brain tissues where glioma cells invade within the brain parenchyma through specific routes that include traveling along the axonal white matter tracts and the peripheral blood vessel basement membranes. It is unclear why glioma cells prefer to invade through these routes but there are two possible reasons that are not mutually exclusive: first, these routes are enriched with certain substrates that support adhesion and stimulate invasion, and second, these routes are also rich sources of oxygen and nutrition. Invasive cells have been found to be different from their non-invasive counterparts genotypically and phenotypically and have been reported to activate a various number of coordinate cellular programs necessary for migration, invasion (Fortin Ensign, Mathews, Symons, Berens, & Tran, 2013; Xie, Mittal, & Berens, 2014a) and survival (e.g. reduced proliferation, marked resistance to apoptosis, upregulation of ATP-binding cassette (ABC)
transporters (Bleau, Huse, & Holland, 2009; Giese, Bjerkvig, Berens, & Westphal, 2003; Joy et al., 2003; Mariani, Beaudry, McDonough, Hoelzinger, Demuth, et al., 2001). Gene expression profiling has identified genes involved in cell invasion and migration that are expressed on a subclass of glioma, and strongly correlate with poor patient survival (Freije et al., 2004; Liang et al., 2005; Li et al., 2009; Phillips et al., 2006; Verhaak et al., 2010). Moreover, and consistent with these phenotypic and genotypic differences, recent genomic profiling studies found that genetic alterations in tumor cells at the invading tumor margin are largely distinct from those found in cells residing in the main tumor mass, and only few percentages of these genetic alterations were shared between the two populations (H. Kim et al., 2015; J. Kim et al., 2015). In addition, the tumor specimens isolated from the tumor mass and invading margins were found to have different immune infiltration and tumor microenvironments (Glas et al., 2010; Pistollato et al., 2010), findings that further highlight the tumor heterogeneity and the need for therapeutic approaches that target all tumor populations. Although the invasive and stem-like glioma disease reservoirs were described here independently, these cell populations are not mutually exclusive and many of the BTICs isolated and characterized to date possess the cardinal features of highly infiltrative glioma.

1.1.6 Intrinsic and extrinsic determinants of glioma invasion

Glioma invasion is not a random process but rather is regulated and guided by a combination of multiple molecular and physical processes along pre-existing tracks of least resistance. Invasive glioma cells travel along white matter tracts (optic radiations, corpus callosum or anterior commissure), adjacent to neurons, and along basement membranes of blood vessels. In order to initiate this process, glioma cells interact with the brain ECM which consists of glycosaminoglycans (GAGs) and a few structural glycoproteins, with the main GAGs being
hyaluronic acid (HA), chondroitin, keratin and heparan sulfates (Giese & Westphal, 1996; Gritsenko, Ilina, & Friedl, 2012). In addition, glioma cells themselves synthesize many ECM components including collagen I, III, IV and VI, tenascin, fibronectin, vitronectin, laminin, hyaluronan, chondroitin sulfate and heparan sulfate proteoglycans (Gritsenko et al., 2012).

A large number of individual proteins have been implicated in glioma adhesion and invasion, that segregate into functional classes including proteins that interact with the ECM and surrounding cellular microenvironment, proteins that degrade the ECM, and proteins involved in glioma cell movement. These proteins include several surface receptors such as integrin αVβ3 (D’Abaco & Kaye, 2007), NCAM (Owens et al., 1998; Prag et al., 2002), CD44 (Gunia et al., 1999; Merzak, Koocheckpour, & Pilkington, 1994; Radotra & McCormick, 1997), BEHAB (Nutt, Zerillo, Kelly, & Hockfield, 2001; H. Zhang, Kelly, Zerillo, Jaworski, & Hockfield, 1998), semaphorins (X. Li, Law, & Lee, 2011; Neufeld et al., 2005), cadherins (Maret et al., 2010; C. Wang et al., 2014), and the ephrin (Eph) receptor-ligand system (Miao et al., 2009; Nakada et al., 2010; Nakada, Drake, Nakada, Niska, & Berens, 2006; Nakada et al., 2004) as well as a number of proteases including the matrix metalloproteinases [MMPs; particularly MMP2 and MMP9 (Kargiotis et al., 2008; Kondraganti et al., 2000; Lakka et al., 2004; W. Wang, McLeod, & Cassidy, 2003)], the ADAMs (a disintegrin and metalloproteinase), plasmin and cathepsins (Gondi, Lakka, Dinh, et al., 2004; Gondi, Lakka, Yanamandra, et al., 2004; Lakka et al., 2004; Rao et al., 1994; Sarkar et al., 2014; Wildeboer, Naus, Amy Sang, Bartsch, & Pagenstecher, 2006) to name a few.

In addition to these intrinsic determinants of glioma invasion, other extrinsic factors have been implicated in glioma invasion. For instance, experimental studies have found that a hypoxic environment within a tumor results in increased glioma cell invasion and migration, and strongly correlates with tumor malignancy (Elstner, Holtkamp, & von Deimling, 2007; Evans et al., 2004);
however, the exact pathophysiological mechanisms and factors underlying hypoxia-induced glioma cell invasion are largely unidentified. Furthermore, chemokines, cytokines and growth factors secreted by tumor-associated macrophages (TAMs) have been shown to not only stimulate the survival and proliferation of glioma cells, but also regulate their migration and invasion (Bettinger, Thanos, & Paulus, 2002; Garris & Pittet, 2013). Moreover, reactive astrocytes have been implicated in increasing glioma cell migration by secreting several factors important in cell migration, including connective tissue growth factor (Halliday & Holland, 2011).

Several experimental techniques have been used to isolate invasive glioma cells including the selection of invasive subpopulations from human cell lines in vitro (Joy et al., 2003; Mariani, Beaudry, McDonough, Hoelzinger, Demuth, et al., 2001), the use of organotypic brain slice cultures (Beadle et al., 2008; Caspani, Echevarria, Rottner, & Small, 2006; Holtkamp et al., 2005; Jung et al., 2001; Jung et al., 1999; Ohnishi, Matsumura, Izumoto, Hiraga, & Hayakawa, 1998) and the collection of tumor and invasive cells from frozen specimens of glioblastoma patients using laser capture microdissection (Hoelzinger et al., 2005; Kislin, McDonough, Eschbacher, Armstrong, & Berens, 2009; Mariani, Beaudry, McDonough, Hoelzinger, Kaczmarek, et al., 2001; Mariani, McDonough, et al., 2001; Toussaint et al., 2012).

Using a novel orthotopic serial in vivo selection procedure (Robbins & Senger, 2013) our laboratory isolated a highly invasive subpopulation of glioma cells and identified the neurotrophin receptor p75NTR as a central mediator of the observed infiltrative behavior (Angela LM Johnston et al., 2007). Functional, biochemical, and clinical studies confirmed that p75NTR significantly increased invasion and migration of genetically distinct glioma cells in vitro and in vivo. Highly invasive glioblastoma patient specimens frequently exhibited robust expression of p75NTR (Angela LM Johnston et al., 2007; L. Wang et al., 2008). In addition, neurotrophin-dependent regulated
intramembrane proteolysis (RIP) of p75NTR was found to be essential for p75NTR-mediated glioma invasion, a process that could be abrogated using clinically relevant γ-secretase inhibitors (L. Wang et al., 2008). Moreover, the composition and rigidity of the tumor-associated ECM was also found to be different between invasive and non-invasive tumors (Alshehri, Robbins, & Senger, 2017).

1.2 Cell autonomous roles of p75 neurotrophin receptor (p75NTR) in glioblastoma progression

1.2.1 Neurotrophins and their receptors: overview

The neurotrophins are a family of proteins that regulate different aspects of neuronal development, function, and survival in both the central and peripheral nervous systems. This family includes nerve growth factor (NGF) (Levi-Montalcini, 1987), brain derived neurotrophic factor (BDNF) (Leibrock et al., 1989), neurotrophin-3 (NT-3) (P. Ernfors, Ibanez, Ebendal, Olson, & Persson, 1990; Maisonpierre et al., 1990) and neurotrophin-4/5 (NT4/5) (Berkemeier et al., 1991; Hallbook, Ibanez, & Persson, 1991). Initially, all neurotrophins are synthesized as 30-35 kilodalton (kDa) precursor polypeptides (proneurotrophins) that can be cleaved by different enzymes to yield (13-16 kDa) mature neurotrophins. In the mature form, these proteins form homodimers held together by hydrophobic interactions (McDonald et al., 1991) that bind and activate specific transmembrane receptors: the p75 neurotrophin receptor (p75NTR) and the tropomyosin-related kinase (Trk) family of tyrosine kinase receptors (TrkA, TrkB and TrkC). These receptors can signal independently or collectively to mediate various biological functions (Reichardt, 2006) and the binding affinity between these individual receptors and each member of the neurotrophin family is variable, providing additional specificity to the receptor-ligand system (Ibanez & Simi, 2012).
In addition to the roles of neurotrophins and their receptors in nervous system development, there is mounting evidence that disturbance of neurotrophins, their receptors or downstream signaling pathways lead to alterations in the normal function of cells. These alterations are not only limited to progression of different neurodegenerative disorders including Parkinson’s disease and Alzheimer’s disease but studies have shown their involvement in various cancers of either neuronal origin, such as gliomas, medulloblastoma and neuroblastoma, or non-neuronal cancers including melanoma, breast, prostate and lung cancers (N. T. Bui et al., 2002; Tomellini, Lagadec, Polakowska, & Le Bourhis, 2014). This thesis focuses on the cell-autonomous “regulating the biological mechanisms in the same cell” and cell non-autonomous “regulating biological mechanisms in neighboring cells” roles of the neurotrophin receptor (p75NTR) in glioma progression.

1.2.2 The neurotrophic receptor p75 (p75NTR)

1.2.2.1 Structure of p75NTR

The p75 neurotrophin receptor, which is also known as CD271, is a type-1 transmembrane receptor and a member of the tumor necrosis factor receptor (TNFR) superfamily that binds to all precursor and mature neurotrophins (Moses V Chao, 2003; Liepinsh, Ilag, Otting, & Ibáñez, 1997). The p75NTR gene contains 5 exons and maps to chromosome 17q21-q22. The gene codes for an mRNA of 3.8 kb, with a 5’ untranslated region of ~300 nucleotides, and an unusually long (~2 kb) 3’ untranslated region (Johnson et al., 1986), which translates into a 427 amino acid polypeptide. The p75NTR protein has a 28 amino acid signal peptide followed by a cysteine-rich region and a serine/threonine-rich region representing the extracellular domain, a transmembrane domain and a relatively short cytoplasmic domain (Johnson et al., 1986). The extracellular cysteine-rich region constitutes the ligand-binding domain and consists of four cysteine-rich
domains (CRDs), each domain contains approximately 40 amino acids, including 6 disulfide
linked cysteines (Baldwin et al., 1992). p75NTR was reported as the first receptor containing these
repeats, a common characteristic that later was identified in most of the receptors in the tumor
necrosis factor (TNF) receptor superfamily (Mallett and Barclay, 1991). The extracellular domain
of p75NTR is highly negatively charged due to its amino acid components, and can be strongly
bound by clusters of positively charged residues in neurotrophins to activate p75NTR signaling
(McDonald et al., 1991; He and Garcia, 2004). The p75NTR intracellular domain has a
juxtamembrane domain that mediates the interaction of p75NTR with several downstream effectors,
and a C-terminal death domain (Large et al., 1989). The p75NTR death domain was identified by
aligning the primary sequence of p75NTR with the intracellular domain sequence of Fas receptor,
a member of the TNF receptor superfamily (Chapman, 1995). Similar to Fas, p75NTR was found to
be able to induce apoptosis (Rabizadeh et al., 1993; Barrett and Bartlett, 1994). Finally, the extreme
end of p75NTR contains three C-terminal amino acids (SPV) that have been shown to be essential
for p75NTR binding with PDZ domain-containing proteins (Irie et al., 1999). Based on the amino
acid sequence of p75NTR, its predicted molecular weight is 50 kDa (Johnson et al., 1986); however,
due to the extensive N- and O-glycosylation of the extracellular domain, the apparent molecular
weight of p75NTR is 75 kDa. Figure 1.2 shows the detailed structure of p75NTR.
The p75 neurotrophin receptor is a type I transmembrane protein. The extracellular domain contains 4 cysteine-rich domains (CRDs), which constitute the neurotrophin binding domain, a single N-linked glycosylation site and a stalk domain which is extensively O-glycosylated. The intracellular domain contains a juxtamembrane region with a palmitoylation site, followed by the death domain. The tail of p75NTR contains the C-terminal amino acids SPV, which is essential for binding with PDZ domain-containing proteins.

Figure 1.2: The structure of the p75 neurotrophin receptor (p75NTR)
1.2.2.2 Expression and Function of $p75^{NTR}$

Initially, it was clear that $p75^{NTR}$ plays a major role in development. $p75^{NTR}$ is a robust marker of neural crest stem cells (NCSCs), a migratory cell population that generates a wide variety of cell and tissue types during embryonic and adult development, including bones, cartilages, connective tissues, pigment and endocrine cells, as well as neurons and glia (Dupin & Sommer, 2012), and was successfully used to isolate NCSCs from fetal and adult tissues (Betters, Liu, Kjaeldgaard, Sundstrom, & Garcia-Castro, 2010). In the brain, $p75^{NTR}$ mRNA was detected in the cerebellum, striatum, medulla, septum and pons in the developing rat and persisted at low levels into adulthood (Patrik Ernfors et al., 1988). In the adulthood, studies showed that $p75^{NTR}$ has a critical function in the synaptic plasticity of the nervous system and is also expressed by different tissues including sensory neurons (Skoff & Adler, 2006), neural progenitors (Kaylene M Young, Tobias D Merson, Areechun Sotthibundhu, Elizabeth J Coulson, & Perry F Bartlett, 2007), mullar glia and retinal cells (Lebrun-Julien et al., 2010). Furthermore, $p75^{NTR}$ expression defines highly proliferative precursor cells isolated from the stem cell niche of the rat subventricular zone (SVZ), which are able to respond to neurotrophin (BDNF and NGF) activation by increasing neuroblast generation, suggesting that these cells are responsible for adult neurogenesis (Kaylene M Young et al., 2007). Moreover, $p75^{NTR}$ was also found to be expressed by different stem cell populations or their progenitors, including bone marrow stem cells, liver stem cells (stellate cells), muscle stem cells (satellite cells), stem cells of the oral and esophageal mucosa, and keratinocytes of the basal layer of the epidermis, of the corneal limbal epithelium and of squamous epithelia (reviewed in (Tomellini et al., 2014)).
p75<sup>NTR</sup> can activate different downstream signaling pathways based on several factors, including cell type and differentiation status, forms and abundance of different neurotrophins, type of p75<sup>NTR</sup> -interacting co-receptor(s), availability of intracellular adaptor molecules, and post-translational modification expression (Lu, Pang, & Woo, 2005). These different p75<sup>NTR</sup> - mediated signaling pathways lead to various cellular responses, including cell death (Coulson et al., 2004), cell survival (Nguyen Truc Bui et al., 2002), neurite outgrowth and retraction (Yamashita, Tucker, & Barde, 1999), regulation of cell cycle (Chittka et al., 2004), myelination (Notterpek, 2003), progenitor differentiation (T. Nakamura, Endo, & Kinoshita, 2007), migration and invasion (Angela LM Johnston et al., 2007; L. Wang et al., 2008). These diverse biological effects can be explained by the ability of p75<sup>NTR</sup> to cooperate with other receptors including, Trk A, Trk B, Trk C, sortilin (SORT1), LINGO-1 and Nogo receptor (NogoR). The pairing of p75<sup>NTR</sup> with Trk receptors facilitates high affinity binding of the different neurotrophins (Hantzopoulos, Suri, Glass, Goldfarb, & Yancopoulos, 1994). Consistent with this, co-expression of p75<sup>NTR</sup> with Trk receptors increases the binding affinity of the mature neurotrophins to the Trk receptors and enhances pro-survival and growth signaling (Hempstead, Martin-Zanca, Kaplan, Parada, & Chao, 1991). Furthermore, p75<sup>NTR</sup> is not only able to modulate the affinity binding of neurotrophins to Trk receptors, but it also enhances their specificity. In the absence of p75<sup>NTR</sup>, TrkA can be activated by both NGF and NT3, while TrkB can be activated by BDNF, NT3 and NT4/5, and TrkC can be activated by NT3. In the presence of p75<sup>NTR</sup>, TrkA is only activated by NGF (Mischel et al., 2001), and TrkB is activated by BDNF (Bibel, Hoppe, & Barde, 1999), but conversely, TrkC can be activated by all neurotrophins (Vesa, Krüttgen, & Shooter, 2000). While the interaction between p75<sup>NTR</sup> and Trk receptors has been linked to cell survival, the interaction of p75<sup>NTR</sup> with sortilin upon proneurotrophin binding induces cell death (Nykjaer et al., 2004; Teng et al., 2005). Finally,
the trimeric complex formed as a result of the interaction of $p75^{NTR}$ with NogoR and LINGO-1 receptors binds to Nogo-66, myelin-associated glycoprotein (MAG), or oligodendrocyte myelin glycoprotein, to inhibit neurite outgrowth by activating RhoA (Mi et al., 2004; Niederost, Oertle, Fritsche, McKinney, & Bandtlow, 2002; K. C. Wang, Kim, Sivasankaran, Segal, & He, 2002).

Expression of $p75^{NTR}$ is upregulated rapidly under a wide range of pathological conditions throughout the nervous system (Ibáñez & Simi, 2012), including ischemia (Kokaia, Andsberg, Martinez-Serrano, & Lindvall, 1998), seizures (Roux, Colicos, Barker, & Kennedy, 1999) spinal cord injury (Brunello, Reynolds, Wrathall, & Mocchetti, 1990), and has been implicated in several neurodegenerative diseases, including Alzheimer’s disease (Chakravarthy et al., 2012), amyotrophic lateral sclerosis (ALS) (Lowry et al., 2001) and multiple sclerosis (Dowling et al., 1999). High expression of $p75^{NTR}$ was also observed in many cancers such as glioma, melanoma and breast cancer.

1.2.3 $p75^{NTR}$ (CD271) as a central regulator of glioma progression

This section will discuss the role of $p75^{NTR}$ in glioma progression a major focus of this thesis. $p75^{NTR}$ regulates several hallmarks of GBM, including invasion and proliferation (J. Berghoff, Jaisimha, Duggan, MacSharry, & McCarthy, 2015; Forsyth et al., 2014; Angela LM Johnston et al., 2007; L. Wang et al., 2008). Invasive glioma cells have a high expression of $p75^{NTR}$ and treating these cells with either mature neurotrophins (NGF, BDNF or NT3) or a proneurotrophin (pro-NGF) that binds to and activates $p75^{NTR}$ but not the Trk receptors, enhances their migration (Angela LM Johnston et al., 2007). In addition, $p75^{NTR}$ ectopic expression in genetically diverse non-invasive glioma cells (U87MG and U251) was found to induce their migration and invasion ability both in vitro and in vivo (Angela LM Johnston et al., 2007) and
these invasive cells showed changes in cytoskeletal rearrangement and a decrease in RhoA activity (Angela LM Johnston et al., 2007). Furthermore, these invading cells, and genetically distinct p75<sup>NTR</sup>-expressing BTICs, were found to express high levels of BDNF that bound to p75<sup>NTR</sup> on the surface, in an autocrine loop that fuels the invasive phenotype (Angela LM Johnston et al., 2007; L. Wang et al., 2008). In agreement with these data, the expression of p75<sup>NTR</sup> mutants crippled for neurotrophin binding abrogated p75<sup>NTR</sup>-mediated glioma invasion <i>in vitro</i>, and expressing cells formed non-invasive, well-circumscribed tumors in orthotopic xenograft models <i>in vivo</i> (Angela LM Johnston et al., 2007). These data confirm that the binding of p75<sup>NTR</sup> to its ligands is required for p75<sup>NTR</sup>-mediated glioma invasion in the context of the glioma models examined thus far, supporting the concept that p75<sup>NTR</sup> on the surface of a glioma cell is constitutively associated with the downstream regulators necessary to mediate glioma invasion. Furthermore, and clinically significant, assessment of p75<sup>NTR</sup> protein expression in a series of glioma patient samples revealed the presence of a p75<sup>NTR</sup>-expressing glioma cell subpopulation in 85% of GBM and 22% of mid-grade astrocytoma; p75<sup>NTR</sup> expressing cells were experimentally confirmed to be more invasive than the non-expressing cells from the same patient (Angela LM Johnston et al., 2007). Moreover, analysis of independent data established by the Repository of Molecular Brain Neoplasia Data (REMBRANDT), the TCGA, and the Human Protein Atlas, confirmed that p75<sup>NTR</sup> is correlated with high-grade glioma, and high expression of p75<sup>NTR</sup> is inversely correlated with overall patient survival (Alshehri et al., 2017).

p75<sup>NTR</sup>, similar to several proteins, including amyloid precursor protein, Notch and ErbB4 receptor tyrosine kinase, has been observed to undergo regulated α-secretase and γ-secretase cleavage, known as regulated intramembrane proteolysis (RIP) (K. C. Kanning et al., 2003; Pardossi-Piquard et al., 2005; Podlesniy et al., 2006). Initially, p75<sup>NTR</sup> undergoes cleavage by a
wide range of metalloproteinases, such as α-secretase, to shed the extracellular domain (ECD), leaving an unstable membrane-bound C-terminal fragment (CTF) that is further cleaved by a presenilin-dependent γ-secretase to release an intracellular domain (ICD) that has potential signaling capacity (DiStefano, Chelsea, Schick, & McKelvy, 1993; Kevin C Kanning et al., 2003). Interestingly, neurotrophin-dependent RIP of p75NTR was essential for p75NTR-mediated glioma invasion, and proteolytic processing of p75NTR was detected in several p75NTR-positive patient tumor specimens and BTICs (L. Wang et al., 2008). Blocking this process by expression of cleavage resistant chimeras of p75NTR or using clinically relevant γ-secretase inhibitors, significantly inhibited the highly invasive nature of genetically distinct glioma cells and patient-derived BTICs in vitro and in vivo, which resulted in prolonged survival in orthotopic xenograft models (L. Wang et al., 2008).

The generation and release of the ICD of p75NTR raised the possibility that it may serve as a messenger for the neurotrophins to both activate an intracellular signal and regulate transcriptional events in the nucleus (M. V. Chao, 2003; Parkhurst, Zampieri, & Chao, 2010). Although the ICD fragment of Notch is involved in a wide range of signaling (Fortini, 2009), less evidence is available about the possible roles of the p75NTR-ICD (A. S. Berghoff, Lassmann, Preusser, & Hoftberger, 2013; L. Wang et al., 2008). p75NTR has been shown to localize within lipid rafts after phosphorylation by PKA. Lipid raft localization is required for downstream signaling in cerebellar neurons (Higuchi, Yamashita, Yoshikawa, & Tohyama, 2003) (Bilderback, Gazula, Lisanti, & Dobrowsky, 1999; Bilderback, Grigsby, & Dobrowsky, 1997; Fujitani et al., 2005; Gil, Cubi, & Aguilera, 2007; Higuchi et al., 2003; C. S. Huang et al., 1999). Disruption of these membrane microdomains has been shown to inhibit p75NTR-mediated invasion (Ahn et al., 2016).

In contrast to the findings that suggest proteolytic processing of p75NTR is required for p75NTR-mediated glioma invasion (L. Wang et al., 2008), p75NTR can also induce glioma migration
*in vitro* through modulation of cadherin-11 (Cdh-11); knockdown of Cdh-11 resulted in a significant inhibition of p75<sup>NTR</sup>-mediated cell migration (J. Berghoff et al., 2015). These findings suggest that p75<sup>NTR</sup> induce glioblastoma cell migration through both γ-secretase-dependent and independent mechanisms, however the full complexity of these diverse signaling paradigms has not been fully understood.

To investigate p75<sup>NTR</sup> downstream signaling pathways that mediate glioma tumor progression, a functional analysis using a series of truncation, deletion and point mutants that disrupt key effector regions of the protein was performed (Ahn et al., 2016). This mutational approach revealed that the extreme C-terminal PDZ-binding motif of p75<sup>NTR</sup> is essential for glioma invasion. A peptide-based affinity strategy using the PDZ-binding motif of p75<sup>NTR</sup>, revealed that PDLIM1, a protein implicated in cell cytoskeleton regulation, was a novel signaling-adaptor for p75<sup>NTR</sup> (Ahn et al., 2016). In highly invasive p75<sup>NTR</sup>-expressing human glioma cells and patient-derived BTICs, PDLIM1 was found to interact with p75<sup>NTR</sup> and down-regulation of PDLIM1 or the complete loss of the p75<sup>NTR</sup> PDZ binding motif (ΔSPV) resulted in significant inhibition of p75<sup>NTR</sup>-mediated glioma invasion (Ahn et al., 2016). Moreover, this study highlighted the regulated nature of this interaction, as prevention of phosphorylation on serine 303, located in the cytoplasmic tail of p75<sup>NTR</sup>, by pharmacological inhibition of the cAMP-dependent protein kinase (PKA), or by a mutational strategy (S303G), crippled p75<sup>NTR</sup>-mediated glioma invasion and resulted in phosphorylation of serine 425 within the C-terminal PDZ-binding motif (SPV). Taken together, all findings discussed above confirmed that p75<sup>NTR</sup> is a central regulator of glioma invasion and is strongly associated with highly invasive cells in GBM tumors. Figure 1.3 illustrates our current working model of p75<sup>NTR</sup> in glioma invasion.
Figure 1.3: The role of p75NTR in glioma invasion

Neurotrophins (NT) binding to p75NTR results in a phosphorylation of the cytoplasmic tail of p75NTR that regulates the interaction of p75NTR with the PDZ domain-containing protein PDLIM1. Preventing phosphorylation of p75NTR by pharmacological inhibition of PKA results in in phosphorylation of serine 425 within the C-terminal PDZ-binding motif (SPV) of p75NTR and inhibition of p75NTR-mediated glioma invasion. Proteases released by glioma cells or other cells in the microenvironment contribute at the ECM degradation and proteolytic processing of p75NTR. p75NTR undergo RIP by sequential cleavage, first by α-secretase and then by γ-secretase. The initial cleavage results in the release of extracellular part of p75NTR leaving a C-terminal fragment (CTF) that remains membrane bound while the later results in the cytoplasmic release of the intracellular domain (ICD). Inhibition of this process by clinically relevant γ-secretase inhibitors abrogated p75NTR-mediated glioma invasion. Whether the release of p75NTR ICD regulates the cytoskeleton arrangement or translocate into the nucleus to regulate transcription of different genes involved in promoting glioma progression is still unclear.
1.2.4 Neurotrophins and their involvement in the glioma microenvironment

The interaction of neoplastic cells with extracellular matrix and other cells in the tumor microenvironment including vasculature, immune cells and normal stromal cells, has been shown to be an important process in tumor formation and progression (Hambardzumyan & Bergers, 2015; Hanahan & Coussens, 2012; Quail & Joyce, 2013; Shiao, Ganesan, Rugo, & Coussens, 2011). The presence of these different cells in their ‘activated’ states within tumors has been implicated in the failure of current therapies and involved in therapeutic resistance (Joyce, 2005; Junttila & de Sauvage, 2013; Ruffell & Coussens, 2015). The unique composition of the glioma microenvironment, which consists of several cell types including neuronal and glial progenitors, neurons, astrocytes, oligodendrocytes, microglia/macrophages and brain endothelial cells, has been found to impact glioma progression by providing factors that promote survival, proliferation, and the invasive behavior of the tumor cells.

Tumor-associated macrophages (TAM) have been linked to high tumor grades, and associated with poor prognosis in many cancers including glioma (Bingle, Brown, & Lewis, 2002; Hussain, Yang, Suki, Aldape, et al., 2006; Hussain, Yang, Suki, Grimm, & Heimberger, 2006; Komohara, Ohnishi, Kuratsu, & Takeya, 2008). The presence of microglia (brain resident macrophages), and infiltrated macrophages throughout glioma tissue was first observed by Wilder Penfield in 1925 (Penfield, 1925). Although the multiple roles of resident microglia and blood borne macrophages in glioma tumorigenesis are controversial, there is substantial evidence on the interactions of these cells within the glioma microenvironment (da Fonseca & Badie, 2013; Wei, Gabrusiewicz, & Heimberger, 2013) including the ability of glioma to generate an immune suppressive environment (Wu et al., 2010; I. Yang, Han, Kaur, Crane, & Parsa, 2010). Depending on the context, microglia/macrophages have pro-tumorigenic (Bettinger et al., 2002; Dziurzynski
et al., 2011; Kerber et al., 2008; Markovic et al., 2009; Platten et al., 2003; Simmons et al., 2011; Zhai, Heppner, & Tsirka, 2011; J. Zhang et al., 2012) or anti-tumorigenic activity (Brantley et al., 2010; Hoa et al., 2007; Hwang et al., 2009; Mora et al., 2009; Nakagawa et al., 2007). Amphotericin B has been used to reprogram microglia/macrophages in glioma tissues so that they inhibit tumor growth, resulting in improvement of overall survival in patient-derived-intracranial (orthotopic) xenograft models (Sarkar et al., 2014). Microglia/macrophages express neurotrophins in vitro and in the cerebral cortex, Globus pallidus, and medulla of the brain (Elkabes, DiCicco-Bloom, & Black, 1996), and stimulation of microglia with lipopolysaccharide (LPS) induces both BDNF and NGF secretion (Nakajima et al., 2001). In addition, protein expression studies have revealed that p75\textsuperscript{NTR} as well as Trk (A, B, C) are expressed by microglia in vitro (Nakajima et al., 1998), and microglia stimulation by neurotrophins results in the phosphorylation of TrkA and TrkB, activation of the NF-κB pathway and increased plasminogen secretion. These effects were inhibited using the protein kinase inhibitor, K252a, suggesting that neurotrophins signaling through Trk receptors regulate microglia function (Nakajima et al., 1998). Furthermore, treating microglia with neurotrophins BDNF, NGF and NT3 enhanced their proliferation and induced their phagocytic activity (Elkabes et al., 1996). Activated microglia also secrete interleukin-6 (IL-6) which induces neurotrophin secretion by astrocytes (Otten et al., 2000), suggesting that neurotrophins can be secreted in the brain as a result of cross-talk between immune cells and brain cells. Collectively, these studies suggest that neurotrophins mediate communication between immune cells and glioma cells in order to promote glioma progression.

1.2.5 A role for p75\textsuperscript{NTR} in cancer: extending beyond glioma

A universal role for p75\textsuperscript{NTR} in carcinogenesis, as either an oncogene or a tumor suppressor, has not been established; however, this is not due to a lack of studies. A possible role for p75\textsuperscript{NTR}
in cancer was observed very early after its initial discovery, when NGF treatment of human melanoma cells, cultured in serum-starved conditions, enhanced their growth and survival (Fabricant, De Larco, & Todaro, 1977). Interestingly, human melanoma cells isolated from a metastatic tumor responded to NGF treatment, but not cells isolated from the primary tumor, suggesting a potential role of p75\textsuperscript{NTR} in the progression of melanoma metastasis (Fabricant et al., 1977). Furthermore, CD271 or p75\textsuperscript{NTR} has been identified as a marker for melanoma-initiating cells (Alexander D Boiko et al., 2010). These cells were capable of establishing tumors and metastases when transplanted into mice (Alexander D Boiko et al., 2010). Brain is rich in neurotrophins and a primary target of melanoma metastasis. Importantly, normal brain tissues surrounding the metastatic melanoma tumors were found to be enriched with NGF, suggesting that neurotrophins in the brain act as chemoattractants for p75\textsuperscript{NTR}-expressing melanoma cells (Menter, Herrmann, Marchetti, & Nicolson, 1993). In addition to melanoma, p75\textsuperscript{NTR} has been found on tumor-initiating cells from breast (Kim et al., 2012) esophageal squamous cell carcinomas (T. T. Huang, Sarkaria, Cloughesy, & Mischel, 2009; Okumura, Shimada, Imamura, & Yasumoto, 2003), hypopharyngeal carcinoma (Imai et al., 2013), squamous cell carcinoma of the head and neck (Murillo-Sauca et al., 2014), neuroblastoma (Biagiotti et al., 2006), medulloblastoma (Morrison et al., 2013) and osteosarcoma (Tian, Li, Si, Liu, & Li, 2014). In many cases its expression has been correlated with poorer clinical outcome. These findings suggest that p75\textsuperscript{NTR} may act as an oncogene in many cancers, however data related to p75\textsuperscript{NTR} biology in both prostate and bladder cancers suggest that p75\textsuperscript{NTR} acts as a tumor suppressor. While p75\textsuperscript{NTR} is highly expressed by normal prostate epithelial cells, p75\textsuperscript{NTR} expression was found to be absent in the early stages of the transformation process (Perez, Regan, Pflug, Lynch, & Djakiew, 1997), and prostate cancer cell lines lack p75\textsuperscript{NTR} expression. Moreover, p75\textsuperscript{NTR}-ectopic expression in prostate cancer cells,
which normally lack p75NTR expression, leads to cell cycle arrest and enhanced apoptotic cell death 
_\textit{in vitro}, and decreased tumor growth and metastasis formation when transplanted into SCID mice (Khwaja, Tabassum, Allen, & Djakiew, 2006; Pflug & Djakiew, 1998). Similarly, ectopic expression of p75NTR in p75NTR-deficient bladder cancer cells led to inhibition of cell proliferation and increased apoptotic cell death (Khwaja & Djakiew, 2003; Arshia Tabassum, Fatima Khwaja, & Daniel Djakiew, 2003). However, in the majority of tumors investigated thus far, as in the case of glioma, p75NTR tends to promote tumorigenesis. The ability of p75NTR to suppress or promote tumor growth continues to highlight the differences in neurotrophin signaling based on cellular context.

1.3 Extracellular Vesicles (EVs): Key regulators of the tumor microenvironment and glioma progression

1.3.1 Introduction

EVs are small phospholipid bilayer-enclosed vesicles secreted by neoplastic and non-neoplastic cells into the extracellular space. Extensive secretion of EVs, along with alteration of cargo contents, has been reported to correlate with various forms of cellular stress such as hypoxia and metabolic reprogramming which occur frequently in tumor microenvironments (Kucharzewska et al., 2013; Park et al., 2010; X. Yu, Harris, & Levine, 2006). Cells can secrete various types of vesicles, which has been challenging for categorization of them in a clear and defined manner (Gould & Raposo, 2013). EVs are broadly sorted based on their size, intracellular origin and biogenesis pathways, into three main categories: exosomes, microvesicles (MVs) and apoptotic bodies (Yanez-Mo et al., 2015). Exosomes are small sized vesicles ranging between 30-200nm in diameter, manufactured within multi-vesicular bodies (MVBs) of the late endocytic tract, and released from cells upon fusion of MVB with the plasma membrane (Raposo et al.,
Microvesicles, also known as cellular microparticles, ectosomes or shedding vesicles, are usually larger than exosomes and range between 200-1000 nm in diameter, and are formed by outward budding and fission of the plasma membrane (Cocucci, Racchetti, & Meldolesi, 2009; Hess, Sadallah, Hefti, Landmann, & Schifferli, 1999; Holme, Solum, Brosstad, Roger, & Abdelnoor, 1994). While exosomes and MVs are released from cells during normal cellular processes, apoptotic bodies are only secreted by cells undergoing programmed cell death. Apoptotic bodies are large vesicles (500-4000 nm in diameter) that are characterized by the presence of cellular organelles as cargo in these vesicles (Akers, Gonda, Kim, Carter, & Chen, 2013). However, in some cases apoptotic bodies are also able to transfer genetic information to recipient cells. For instance, it has been reported that uptake of apoptotic bodies released from H-rasV12 or human c-myc-transfected cells by murine fibroblasts led to loss of contact inhibition of murine fibroblast in vitro, is a phenotype of proliferative cancer cells, and a tumorigenic phenotype in vivo (Bergsmedh et al., 2001).

Categorizing vesicles based on their cellular origin or their sizes remains problematic due to the overlap in the sizes of the different types of vesicles and the lack of a complete understanding on the cellular biogenesis of each type. Despite all current challenges and limitations in selectively isolating and characterizing one EV population from another, it is now well established that a single cell can release different types of EVs. Although the current isolation and purification methods often result in mixtures of heterogeneous EV populations, there is increasing evidence that those EVs are not only artifacts of purification, they are released from many –if not all- cells in a well-regulated manner to mediate a novel way of communication between cells through exchange of structural proteins, RNA, miRNA and lipids. In addition, EVs have been shown to travel through body fluids to distant organs in order to convey functional information both in
Physiological and pathological conditions (Gross, Chaudhary, Bartscherer, & Boutros, 2012; Peinado et al., 2012).

Studying the role of EVs in different cancers and neurological disorders has recently been the focus of many scientists for two reasons. The first reason is that the discovery of specific biological roles mediated by EVs may significantly advance our understanding about the pathogenesis, progression and in some cases the relapse of different diseases. Another reason is that a detailed biochemical, functional and molecular characterization of different types of EVs populations might lead to the identification of accurate diagnostic and prognostic biomarkers of different cancers.

1.3.2 Biogenesis of EVs and cargo selection

Exosomes are a subtype of EVs that can be defined by various physical and morphological characteristics. First of all, exosomes are small and sediment at 100,000 g and have 1.13-1.19 g/ml density on a sucrose gradient (Xu, Greening, Zhu, Takahashi, & Simpson, 2016). Morphologically, exosomes have spherical structures defined by a lipid bilayer when visualized under an electron microscope (Raposo et al., 1996). Different receptors, nucleic acids, and proteins that are either contained within the aqueous core or in the lipid membrane of exosomes, have been found to reflect the cell of origin (Lo Cicero, Stahl, & Raposo, 2015).

The biogenesis of exosomes through the maturation of the cell endosomal system starts with early endosomes that mature into late endosomes or MVBs that invaginate their membranes to generate intraluminal vesicles (ILVs). After maturation, MVBs fuse with the cell membrane and release exosomes extracellularly (Kowal, Tkach, & Thery, 2014). This biogenesis process can be either dependent or independent of the “Endosomal Sorting Complex Required for Transport” (ESCRT) (Kowal et al., 2014). ESCRT-dependent mechanisms act on MVBs, resulting in a
selective sorting of particular receptors and proteins into ILVs (Raiborg & Stenmark, 2009). The ESCRT family includes four different protein complexes; ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III. ESCRT-0 can identify ubiquitinylated proteins protruding into the MVB membrane then separate them into specific micro-domains. ESCRT-0 then binds to ESCRT-I, which in turn recruits the ESCRT-II complex, resulting in inward budding of ILVs into MVBs. During this internalization step, various cytosolic proteins and RNAs have direct access into the forming vesicles (Colombo et al., 2013). Next, the ESCRT-III subunit is recruited by the ESCRT-II complex into the neck of the budding ILVs, resulting in their cleavage from the inner membrane of the MVB and release as free vesicles inside MVBs. The MVBs (late endosomes) then fuse with cell membrane and release exosomes into the extracellular space (Raiborg & Stenmark, 2009).

Conversely, several reports have shown that MVB biogenesis and exosome release can happen independent of the ESCRT machinery, and despite simultaneous silencing of all four ESCRT-complexes, ILVs are still developed in the MVBs, suggesting the presence of alternative mechanisms for the biogenesis of exosomes (Stuffers, Sem Wegner, Stenmark, & Brech, 2009). For instance, tetraspanins, transmembrane proteins enriched in exosomes, have been shown to be involved in exosome release through an independent ESCRT pathway (Chairoungdua, Smith, Pochard, Hull, & Caplan, 2010; Hurwitz, Conlon, Rider, Brownstein, & Meckes, 2016; Nazarenko et al., 2010). Ectopic expression of tetraspanin CD82 and CD9 enhanced the exosomal release of β-catenin from HEK293 cells, while bone marrow dendritic cells (BMDCs) isolated from CD9 knockout mice secrete fewer exosomes compared to BMDCs isolated from CD9 wild-type mice (Chairoungdua et al., 2010).

Recently, the tetraspanin CD63 was shown to regulate exosome biogenesis and CRISPR/Cas9 knockout of the CD63 gene in HEK293 cells was associated with a significant
reduction of exosome release, suggesting the importance of CD63 exosome biogenesis (Hurwitz et al., 2016).

Lipids are also important regulators of vesicular transport and are involved in exosome biogenesis. Trajkovic et al., have shown that inhibition of neutral sphingomyelinase 2 (nSMase2), an enzyme that generates ceramide from sphingomyelin, reduced exosome release (Trajkovic et al., 2008). The author used an oligodendrocyte cell line (myelinating cells of the central nervous system) to also study the role of proteolipid protein in the formation and release of exosomes. Proteolipid protein is essential for forming myelin, the lipid-rich membrane that oligodendrocytes use to enwrap and insulate axons. Disrupting the expression of nSMase2 by using either specific inhibitors or RNA interference reduced secretion of proteolipid protein-containing exosomes in a mechanism that does not require ESCRT machinery (Trajkovic et al., 2008). Phospholipase D2 (PLD2), an enzyme that produces phospatidic acid (PA) from phospholipids, is another lipid modifier enzyme that has been found to correlate with the number of exosomes released (Laulagnier et al., 2004). Although exosome biogenesis has been associated with ESCRT-dependent or ESCRT-independent biogenesis machineries, these pathways may not be completely separated but instead might get activated synergistically to release different types of EVs.

The mechanisms for MVs biogenesis and budding from the plasma membrane are still largely un-discovered. The number of MVs–shedding from the plasma membrane of breast cancer cells has been shown to increase with the activation of actin-myosin machinery and small GTPases, such as ADP-ribosylation factor 6 (ARF6) (Muralidharan-Chari et al., 2009). Moreover, Nabhan et al., (2012) showed that the budding of MVs is driven by a specific interaction of ESCRT-I subunit tumor susceptibility gene 101 (TSG101) with a tetrapeptide
PSAP motif of an accessory protein, arrestin domain-containing protein 1 (ARRDC1), which is localized to the plasma membrane through its arrestin domain. This interaction leads to TSG101 relocation from endosomes to the plasma membrane and promotion of the budding of MVs containing TSG101 and ARRDC1, along with other proteins (Nabhan, Hu, Oh, Cohen, & Lu, 2012). Collectively, these findings suggest that the molecular machineries for both exosomes and MVs may share major elements, however, those molecular machineries might be cell-type and cell-context dependent, which eventually result in biogenesis and release of heterogeneous populations of EVs.

1.3.3 The role of EVs in the central nervous system (CNS)

Different cells in the CNS, including neurons, oligodendrocytes, astrocytes and microglia have been reported to release EVs into the extracellular environment. These EVs have at least two main functions: signaling to neighboring cells to mediate transfer of various biomolecules and to dispose of unneeded cell components (Simons & Raposo, 2009).

1.3.3.1 The role of EVs in the development of the CNS

During the early development and neurogenesis of the mouse brain, a massive number of EVs containing prominin-1 (CD133), a pentaspan membrane protein known as a marker for neural stem and progenitor cells, have been reported to be secreted into the ventricular fluid in the neural tube; however, the role of these vesicles is still unidentified (Marzesco et al., 2005). Another study suggested that these EVs may play a major role in transferring mRNA encoding pluripotent transcription factors which can reprogram phenotypes of other cells to support development (Ratajczak et al., 2006). Oligodendrocyte-derived EVs have been shown to involved in the inhibition of both the morphological differentiation of oligodendrocytes and myelin formation.
The secretion of these auto-inhibitory EVs are controlled by neurons to coordinate myelin membrane biogenesis (Bakhti, Winter, & Simons, 2011).

In *Drosophila*, several studies have highlighted the importance of EVs in different developmental stages. For instance, Wnts were shown to be secreted in EVs from both human cells and during drosophila development and these vesicles travel away from donor cells to induce Wnt signaling pathway in distant target cells, a process that requires the R-SNARE Ykt6 as a component for exosomal Wnt secretion in cell culture and *in vivo* (Gross et al., 2012). Another population of EVs known as argosomes, which are membrane exovesicles secreted by drosophila imaginal disc epithelium, were found to act as vehicles that transport a morphogenic Wnt signaling protein along temporal and spatial gradients in wing development (Greco, Hannus, & Eaton, 2001). Furthermore, EVs were also shown to transport the Wnt-binding protein Evenness Interrupted/Wntless/Sprinter (Evi/Wls/Srt) at the neuromuscular junction (NMJ) of *Drosophila* both during development and in mature neurons (Korkut et al., 2009). Taken together, all these findings suggest that EVs contribute to the development of drosophila through transporting and activating Wnt in different organs. Similar functions in humans have not yet been investigated.

Hedgehog (Hh) proteins, which play major roles during embryonic tissue development, have also been found secreted in EVs from *Drosophila* wing imaginal discs through a process that requires the ESCRT complex. These EVs carry pools of Hh and ESCRT proteins into the extracellular space *in vivo* which can subsequently be detected together at the surface of receiving cells, revealing a new route for the transport of secreted Hh across the tissue to achieve a long-range target induction of Hh signaling (Matusek et al., 2014). Taken together, all above
studies suggest that EVs are playing major role in development through transporting essential elements required for activating signaling pathways in distant cells.

1.3.3.1.1 The role of neuronal EVs

EVs secreted by neurons have been implicated in synaptic plasticity. Both cultured embryonic and mature mammalian neurons secrete EVs in response to different factors such as GABA receptor blockers and the application of Ca\(^{2+}\) ionophores (Chivet et al., 2014; Faure et al., 2006). Upon stimulation by depolarization, undifferentiated cortical neurons release EVs containing L1, a neuronal cell adhesion protein, and GluR2/3 subunits of glutamate AMPA receptors (Faure et al., 2006). These findings suggest that release of EVs from different neurons may help them to adapt to various stimuli through the efficacy of synaptic transmission by depletion of neurotransmitter receptors.

1.3.3.1.2 The role of oligodendrocytes EVs

Oligodendrocytes, glial cells that myelinate axons in the brain, secrete EVs containing myelin proteolipid protein (PLP or lipophilin), a myelin protein responsible for maintenance of myelin, in response to the calcium-ionophore ionomycin, suggesting that oligodendrocyte-derived EV secretion is regulated by cytosolic calcium levels. These EVs have been implicated in axon protection against oxidative stress (Kramer-Albers et al., 2007). In addition, EV release from oligodendrocytes is also triggered by the neurotransmitter glutamate through activation of ionotropic glutamate receptors. These EVs are then internalized by neurons via an endocytic pathway, and the EVs cargo is recovered by target neurons in culture, as well as in vivo after injection of EVs into the mouse brain. Furthermore, neurons treated with oligodendroglial EVs were protected when challenged with stressful growth conditions, including oxygen and glucose deprivation (Fruhbeis et al., 2013), suggesting that the signal mediated by EV transfer from
oligodendrocytes to neurons contributes to the preservation of axonal health. The release of these vesicles from oligodendrocytes is regulated by Rab35 activity as the inhibition of Rab35 function in the oligodendrocyte precursor cell line Oli-neu, resulted in intracellular accumulation of endosomal vesicles and inhibition of EVs secretion (Hsu et al., 2010).

Oligodendrocyte-derived EVs were also shown to be specifically and efficiently taken up by microglia both in vitro and in vivo. Internalization of these EVs happens through a macropinocytotic process without inducing a concomitant inflammatory response and microglia that preferentially internalized these EVs do not seem to have antigen-presenting capacity (Fitzner et al., 2011), suggesting that the continuous macropinocytotic clearance of oligodendrocyte-derived EVs by a subpopulation of microglia represents an essential mechanism by which microglia contribute to the oligodendroglial EV cargo in an immunologically 'silent' manner.

1.3.3 The role of astrocyte EVs

Astrocytes are multifunctional interactive cells and part of blood brain barrier (BBB). They play a major role in regulating the ionic balance and providing trophic support for neurons during developmental or pathological conditions. Astrocytes also secrete EVs that carry IL-1beta, a cytokine involved in CNS inflammatory events. The secretion of these EVs is triggered by ATP binding to P2X7 receptor and associated with rapid activation of acid sphingomyelinase, which moves to the outer leaflet of the plasma membrane (Bianco et al., 2009). The release of these EVs was significantly reduced by inhibiting acid sphingomyelinase, and was completely blocked in cultured glial cells isolated from acid sphingomyelinase knockout mice (Bianco et al., 2009). Astrocytes also secrete EVs that contain synapsin 1, a protein expressed in nervous tissue of vertebrates and invertebrates, and classically associated with synaptic vesicles and implicated in neural development (S. Wang et al., 2011). Recruit cultured astrocytes to either oxygen/glucose
deprivation or hydrogen peroxide or incubating astrocyte-derived EVs in high KCl concentrations resulted in release of synapsin I from EVs. Synapsin I interacts with neural cell adhesion molecule 1 (NCAM1) in an oligomannose-dependent manner to promote neurite outgrowth and neuronal survival (S. Wang et al., 2011). Taken together, the findings described above suggest that EV released by astrocytes contain essential elements that are directly or indirectly involved in neurite outgrowth and neuronal survival, repair and protection from cellular stress.

1.3.3.1.4 The role of microglia-derived EVs

Microglia are the resident macrophages in the brain that are important in clearing infections and contribute to repair of brain injury. Similar to astrocytes, microglia secrete EVs in response to ATP released from astrocytes in the absence of cell damage, or by dying cells at sites of brain injury. These EVs also contain IL-1beta, the key initiator of acute inflammatory response. IL-1β efflux from EVs was enhanced by ATP stimulation and inhibited by pretreatment with the P2X7 antagonist oxidized ATP (Bianco et al., 2005), thus indicating crucial involvement of the EVs in the crosstalk between astrocytes and microglia during brain injury and/or inflammation.

As a novel mechanism of communication between microglia and neurons, microglia-derived EVs can stimulate synaptic activity. The uptake of microglia-derived EVs by cultured rat hippocampal neurons or the injection of these EVs directly to the visual cortex of rats, results in an increase in synaptic vesicle release at presynaptic terminals and an increase in ceramide and sphingosine production in neurons, which can be prevented by pharmacological or genetic inhibition of sphingosine synthesis (Antonucci et al., 2012). Consistent with these findings, sphingosine was also shown to induce synaptic vesicle exocytosis through activation of synaptobrevin in synaptic vesicles to form the SNARE (soluble-N-ethylmaleimide sensitive
factor attachment protein receptor) complex (Darios et al., 2009). Although the active molecule(s) within the microglia-derived EVs that induce synaptic vesicle release is still unidentified, these EVs are emerging as a novel way by which microglia regulate synaptic activity and highlight the role of neuronal sphingosine in this microglia-to-neuron communication. In addition, microglia-derived EVs have been found to play an indirect role in development and homeostasis of neuronal synapses by transporting active endocannabinoids, which regulate neuronal synaptic communication within the CNS (Gabrielli et al., 2015). These EVs carry on their surface N-arachidonoylethanolamine (AEA), which is able to stimulate type-1 cannabinoid receptors (CB1) on cultured inhibitory neurons and suppress spontaneous release of GABA (Gabrielli et al., 2015); however, microglia-derived EVs seem to carry another molecule(s), which is able to activate synaptic activity, as blocking CB1 did not inhibit the increase in glutamatergic transmission elicited by microglia-derived EVs (Antonucci et al., 2012; Gabrielli et al., 2015). Taken together, the observations discussed above indicate that microglia-derived EVs are playing an important role in cell-cell communication the CNS.

1.3.4 Extracellular vesicles as biological regulators of glioma progression

Glioma cells secrete different populations of EVs. These EVs have been shown to mediate tumor growth, invasion, migration, angiogenesis, chemoresistance and survival against host immune responses, as well as modulating the metabolic status of different cells in the tumor microenvironment (reviewed in (Chistiakov & Chekhonin, 2014; D'Asti, Chennakrishnaiah, Lee, & Rak, 2016; Nakano, Garnier, Minata, & Rak, 2015). In addition to cancer cells, the microenvironments of both low-grade glioma and high-grade glioma have been found to host non-tumorigenic multipotent stem cells that enhance the invasive behavior of glioma initiating cells through released EVs (Bourkoula et al., 2014). Although these glioma- associated stem cells need
more characterization and understanding of their roles in glioma progression, it is clear they communicate with other glioma cells through EVs to increase the aggressiveness of glioma tumor progression.

The role of EVs in glioma progression is an important area to study for several reasons. First, these tumors are extremely heterogeneous, as genomic profiling studies have illustrated the presence of at least six molecular subtypes of high grade gliomas (Brennan et al., 2013; Noushmehr et al., 2010; Verhaak et al., 2010). Several oncogenic signatures and drivers of these different subtypes are associated with the increase of EVs biogenesis and release (Al-Nedawi et al., 2008; Skog et al., 2008). More importantly, glioma-derived EVs can be used as a valuable source of potential tumor-associated diagnostic and prognostic biomarkers that can be obtained from glioma patient fluids without using invasive procedures. Finally, these EVs can be modified to work as a new potent tool for targeted delivery of anti-cancer drugs.

### 1.3.4.1 The role extracellular vesicles in proliferation of glioma cells

Glioma-derived EVs are enriched with many oncogenic drivers that have been implicated in proliferation of tumor cells and the progression of glioma. One of these oncogenic drivers is the EGFR variant III (EGFRvIII), which lacks 267 amino acids from the extracellular domain, resulting from a deletion of exons 2-7 of the EGFR gene, forming a truncated receptor that is able to constitutively conduct ligand-independent signaling. Aberrant EGFRvIII signaling is crucial in driving tumor progression and often correlates with poor prognosis. EGFRvIII was detected in a considerable proportion (an overall frequency of 25–64% when assessed by multiple techniques in the same tumor) of GBM patients (Gan, Cvrljevic, & Johns, 2013). Furthermore, the phosphorylated and bioactive mutant EGFRvIII oncoprotein and EGFRvIII mRNA were found to be secreted in glioma-derived EVs by different research groups (Al-Nedawi et al., 2008; Skog et
al., 2008). Skog et al, showed firstly that the EGFRvIII mRNA is a cargo in glioma-derived EVs isolated from primary GBM cell cultures and in the serum EVs of 7 out of 25 GBM patients diagnosed with EGFRvIII-positive tumors (Skog et al., 2008). Importantly, EGFRvIII was not detected in EVs isolated from the serum of those patients after surgical resection of the GBM tumors (Skog et al., 2008), suggesting that viable cancer cells with EGFRvIII were the source of these EVs and highlight the idea of using these EVs as diagnostic biomarkers that could help in making the best therapeutic decision for cancer patients through a blood test. In another study, EGFRvIII was shown to be transferred between glioma cells through EVs, and also present in EVs isolated from serum of tumor-bearing mice. The ectopic expression of EGFRvIII in glioma cells increased EGFRvIII-containing EV release, and subsequent fusion with the plasma membranes of cancer cells lacking EGFRvIII. This induced several oncogenic activities including changes in expression of EGFRvIII-regulated genes (VEGF, Bcl-x(L), p27), activation of transforming signaling pathways (MAPK and Akt), morphological transformation and increase in anchorage-independent growth capacity (Al-Nedawi et al., 2008). These findings suggest that glioma-derived EVs can contribute to horizontal propagation of oncogenes and mediate a transforming phenotype among at least a subpopulation of glioma cells. The observation that EGFRvIII is in EVs of GBM patients was confirmed by different studies that also found it in EVs isolated from the cerebrospinal fluid and blood of GBM patients (Figueroa et al., 2017; Graner et al., 2009; Santiago-Dieppa et al., 2014).

Amplification or mutation of the platelet-derived growth factor receptor (PDGFR) in human GBM have revealed a causative role of the PDGF receptor pathway in glioma initiation and progression. Proteomic analysis of glioma cell derived EVs showed that PDGFR-α was present in these EVs (Shao et al., 2012) and the uptake of EVs isolated from cells grown under hypoxic
conditions, resulted in activated PDGFR-α in recipient cells, promoting their growth (Kucharzewska et al., 2013). Although the exact functions of PDGR-containing EVs have not been identified, these findings suggest that PDGR-containing EVs may contribute in glioma progression.

Genetic alteration of the tumor suppressor PTEN, is one of the most common mutations in GBM, occurring in around 70% of cases and associated with the mesenchymal subtype (Verhaak et al., 2010). PTEN governs a wide range of cellular processes including proliferation, survival, energy metabolism and cellular architecture by suppressing the phosphoinositide 3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) pathway through its lipid phosphatase activity. PTEN is exported through EVs that are then internalized by recipient cells, resulting in reduction of serine and threonine kinase Akt phosphorylation, and inhibition of cellular proliferation (Putz et al., 2012). Although no study so far has found a non-cell-autonomous oncogenic effect resulting from intercellular transfer of mutated PTEN through EVs in glioma, it has recently been shown that astrocytes in the brain microenvironment release EVs loaded with microRNAs that selectively downregulate PTEN in breast cancer cells metastasized to the brain, and blockade of astrocyte exosome secretion rescued the PTEN loss, and suppressed brain metastasis in vivo (L. Zhang et al., 2015). These findings highlight the dynamic and reciprocal cross-talk between tumor cells in the microenvironment, suggesting that PTEN can be epigenetically downregulated by specific microRNAs transported in EVs. Furthermore, proteomic analysis of EVs isolated from patient-derived glioma stem cells of either proneural or mesenchymal subtypes showed that the EV signatures were heterogeneous between the two subtypes, and reflected the molecular make-up of glioma stem cells (Ricklefs et al., 2016). Importantly, transfer of EVs derived from the mesenchymal glioma stem cells, which are clinically correlated with poor survival, into proneural
glioma stem cells increased their proliferation capacity *in vitro* and enhanced their tumorigenic behaviors *in vivo* (Ricklefs et al., 2016) indicating that heterogeneity within GBM tumors is propagated at least in a part via EV communication, enhancing the aggressive behaviors of these tumors.

Downregulation of Chloride Intracellular Channel-1 (CLIC1), which is functionally active as a chloride channel in glioma stem cells, either genetically or by using a blocking antibody, inhibited glioma stem cell proliferation and self-renewal *in vitro* and reduced tumorigenic behavior of these cells *in vivo* (Setti et al., 2013). CLIC1 is released in EVs by glioma cells, including glioma stem cells (Setti et al., 2015). Treatment of glioma cells with CLIC1-containing EVs stimulates cell growth both *in vitro* and *in vivo* in a CLIC1-dose dependent manner (Setti et al., 2015), suggesting that in addition to the cell-autonomous roles of CLIC1 in promoting the self-renewal and proliferation of glioma stem cells, CLIC1-containing EVs can mediate similar effects in a non-cell- autonomous mechanism.

In addition to oncoproteins and mRNA, several oncogenic miRNAs involved in glioma progression can be transported by glioma-derived EVs, including miRNA-21 (C. C. Li et al., 2013; Skog et al., 2008). Chan *et al.* demonstrated that miRNA-21 was upregulated in several glioma cell lines and human tumors and downregulation of miRNA-21 inhibited cell survival by inducing apoptosis via caspase 3/7 (Chan, Krichevsky, & Kosik, 2005). Glioma-derived EVs are highly enriched with miRNA-21 with a more than 22-fold increase in miRNA-21 compared to other miRNAs in these EVs (C. C. Li et al., 2013). Moreover, miRNA-21 has been identified in the EVs isolated from GBM patients (Skog et al., 2008), suggesting that the presence of miRNA-21 in glioma-derived EVs may have a pathological function during the glioma progression that has yet to be identified.
1.3.4.2 The role extracellular vesicles in invasion and migration of glioma cells

Migration and infiltration of glioma cells throughout the brain is a hallmark of GBM that hinders successful surgical resection and fuels the recurrence of GBM tumors. Tropomyosin-related kinase B (TrkB), which has been shown to be sufficient to transform a neural crest-derived cell line into a malignant phenotype (Dewitt et al., 2014), is released in EVs isolated from plasma of glioblastoma patients and in EVs derived from glioma cells. YKL-40, also known as chitinase-3-like-1 or human cartilage glycoprotein-39, has been shown to involve in ECM degradation, cell migration, proliferation, survival of GBM and used as a serum potential marker for GBM (Pinet et al., 2016). TrkB-containing EVs can promote invasion and proliferation of YKL-40-inactivated glioma cells, (Pinet et al., 2016). In another study, neural adhesion/recognition protein L1 (L1CAM also known as CD171), an ECM component implicated in induction of tumor cell motility in several tumors including high-grade gliomas, was found to be released in glioma-derived EVs and correlated with more invasive behavior when cleaved by ADAM10, a member of the disintegrin and metalloprotease (ADAM) family, which was also found in glioma derived-EVs (M. Yang et al., 2011). Heparan sulfate proteoglycans (HSPGs), which also exist at the cell surface and in the ECM, have been associated with glioma cell invasion and migration, can function as internalizing receptors of glioma cell-derived EVs. EVs-mediated stimulation of glioma cell migration was significantly reduced in PG-deficient mutant cells, or by treatment of glioma cells with heparin or xyloside, an inhibitor of proteoglycan biosynthesis (Christianson, Svensson, van Kuppevelt, Li, & Belting, 2013). Glioma cell-derived EVs could also contribute to the activation of cell migration and invasion by delivering matrix metalloproteinases (MMPs) to the recipient cells. There is increasing evidence that MMPs contribute to glioma cell invasion into the surrounding normal
tissues through cell-surface ECM degradation (Nakada, Okada, & Yamashita, 2003). Furthermore, EVs derived from GBM cells grown under stress conditions enhanced the migration of recipient cells, and their molecular profiling revealed an abundance of molecules related to signaling pathways important for cell migration (Arscott et al., 2013; Kucharzewska et al., 2013). For instance, it has been shown that glioma cells grown under hypoxic conditions release EVs enriched with MMPs, particularly MMP9, and the uptake of these EVs mediated secretion of several potent growth factors and cytokines, stimulating migration of recipient cells (Kucharzewska et al., 2013). In addition, ionizing radiation has been associated with increases in EV release by glioblastoma cells and normal astrocytes, and EVs-derived from irradiated cells enhanced the migration of recipient cells through the activation of several molecules involved in cell migration, including focal adhesion kinase, Paxillin, and proto-oncogene tyrosine-protein kinase Src (Arscott et al., 2013). These findings suggest that glioma cells communicate through EVs to escape growth stress conditions such as hypoxia and ionizing radiation, through enhancing the invasive behavior of EV recipient cells.

Glioma-derived EVs can transfer different miRNAs that regulate invasiveness of recipient cells (C. C. Li et al., 2013; Skog et al., 2008). Van der Vos and colleagues have found that glioma derived-EVs interact in vivo with microglia/macrophages to deliver functional miR-21 and miR-451. Microglia/macrophages uptake of glioma derived-EVs resulted in increased levels of both microRNAs, increased proliferation, and shifted their cytokine profile toward immune suppression, resulting in more aggressive and invasive tumors (van der Vos et al., 2016). On the other hand, miR-1 also has anti-tumorigenic functions, and down-regulation of miR-1 leads to upregulation of several target genes involved in glioblastoma growth and invasion. The ectopic expression of miR-1 in glioma stem cells blocked their EV-mediated
invasiveness, sphere formation, and angiogenesis (Bronisz et al., 2014), suggesting that glioma derived-EVs can be engineered to act against the glioma cells reducing their invasive behavior and growth. Collectively, all findings discussed above suggest that glioma-derived EVs can regulate the glioma invasion and contribute to glioma progression.

1.3.4.3 The role of glioma derived EVs in angiogenesis

Angiogenesis is a major event in the progression of GBM and among all solid tumors, GBM has been reported to be the most angiogenic, displaying the highest degree of vascular proliferation and endothelial cell hyperplasia (Brem, Cotran, & Folkman, 1972). Several mechanisms have been associated with the robust formation of blood vessels observed in these tumors. One is the sprouting of capillaries from pre-existing blood vessels, which has been correlated with the presence of hypoxia in the tumor core. The release of angiogenic factors from tumor cells helps in recruiting other cells that participate in vessel formation (Das & Marsden, 2013). This can occur by packaging angiogenic factors in EVs. For instance, Taraboletti et al., 2006 and Skog et al. 2008, have shown that glioma and ovarian cancer cells release EVs enriched with high concentrations of soluble angiogenic molecules such as interleukin 8 (IL-8), the vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) (Skog et al., 2008; Taraboletti et al., 2006). Uptake of these EVs stimulated endothelial cell motility and proliferation, confirming the role of glioma-derived EVs in angiogenesis. Furthermore, U251, the human glioma cell line, can release EVs containing different pro-angiogenic factors, including VEGF, the transforming growth factor β type 1 (TGF-β1), and the C-X-C chemokine receptor type 4 (CXCR4), along with plasminogen activators and several MMPs (Giusti et al., 2016). Treatment of human brain microvascular endothelial cells with U251-derived EVs promoted their motility, proliferation, and tube
formation (Kucharzewska et al., 2013). In addition, human brain microvascular endothelial cells treated with these EVs exerted enhanced paracrine stimulation of pericytes, which contribute to GBM progression through stabilization of the proliferative vasculature, as well as enhanced paracrine stimulation of GBM cells, resulting in their increased proliferation and migration (Kucharzewska et al., 2013). Furthermore, CXCR4-containing EVs released by glioma cells were able to activate endothelial cells and induce their tube formation (Bronisz et al., 2014). Downregulation of CXCR4 with siRNA or blocking CXCR4 function by a chemical antagonist in glioma stem cells abrogated their tumorigenic capacity in vivo and diminished angiogenesis (Ping et al., 2011). Another study reported that activated EGFR-containing EVs released by cancer cells are able to induce VEGF expression in endothelial cells and activate autocrine signaling of its key receptor (VEGF receptor-2) resulting in increased tumor growth and microvascular density (Al-Nedawi, Meehan, Kerbel, Allison, & Rak, 2009). These findings suggest that oncogene-containing tumor cell-derived EVs could serve as angiogenesis-modulating stimuli and are able to switch endothelial cells to act in an autocrine mode.

Several miRNAs act as regulators of GBM vascular development and are suggested as potential therapeutic agents since they can be used as inhibitors of tumour angiogenesis (Khorshidi, Dhaliwal, & Yang, 2016). EVs derived from CD133 positive glioma stem cells are enriched with miR-21 and can induce the angiogenic ability of endothelial cells through a miR-21/VEGF signal, an effect that was inhibited by siRNA targeting VEGF (X. Sun et al., 2017). Other miRNAs in glioma derived EVs, including miR-29a, miR-30e and miR-296, reportedly act as stimulators of angiogenesis and are involved in regulating the endothelial cell response in brain tumors (C. C. Li et al., 2013; Wurdinger et al., 2008). In addition to miRNAs, glioma derived EVs have been shown to contain long non-coding RNAs, non-protein coding transcripts
that regulate expression of several genes at epigenetic transcriptional and post-transcriptional levels (Lang et al., 2017). A long non-coding RNA, CCAT2, was found to be upregulated in glioma tissues and in EVs released by glioma cells, and is associated with several tumorigenic phenotypes of glioma cells, including increased cell proliferation, cell cycle progression and migration (Guo et al., 2016; Lang et al., 2017). Uptake of these EVs by human umbilical vein endothelial cells (HUVECs) in vitro promoted their migration, proliferation, tube-like structure formation, and inhibited apoptosis induced by hypoxia, and triggered arteriole formation in vivo. (Lang et al., 2017).

Endothelial cells are main components in forming blood vessels, and can release EVs that promote angiogenesis in vitro and in vivo (Deregibus et al., 2007). EVs derived from endothelial progenitor cells were found to incorporate into endothelial cells by interaction with α4 and β1 integrins expressed on the EV surface, and subsequently activate several angiogenic events. The incorporation of EVs into endothelial cells promoted their cell survival, proliferation, and organization in capillary-like structures in vitro and in SCID mice, while EV-stimulated human endothelial cells organized in patent vessels (Deregibus et al., 2007). Taken together, all findings discussed above suggest that different cells in glioma microenvironments, including both transformed and non-transformed cells, release EVs containing angiogenic factors that promote tumor growth and enhance angiogenesis.

1.3.4.4 The role of glioma derived EVs in chemoresistance

GBM remains a tumor with a dismal prognosis due to the failure of current treatment regimes. Glioblastoma stem cells positive for CD133 showed a strong capability to survive chemotherapy (Liu et al., 2006), and may be responsible for glioma recurrences that invariably result in patient death. Intrinsic and acquired chemoresistance is involved in the treatment
failure in glioma patients. Alkylating agents, including temozolomide (TMZ), the gold standard chemotherapeutic drug for GBM palliative treatment, damage DNA and induces apoptosis, but the cytotoxic activity of these agents is mainly dependent on DNA repair pathways. The DNA repair enzyme O6-methylguanine methyltransferase (MGMT), which can eliminate the cytotoxic O6-methylguanine DNA adduct before it induces DNA damage, is key in developing resistance to TMZ. GBM patients with a methylated MGMT promoter benefited from TMZ, whereas those with an unmethylated MGMT promoter did not benefit from the same agent (Hegi et al., 2005). Another mechanism of resistance to alkylating agents is a deficiency in the functional mismatch repair (MMR) pathway (Hickman & Samson, 1999). In addition, certain cancer cells can expel drugs using specialized transporters of the multi-drug resistance (MDR)-ATP binding-cassette transporter (ABC transporters) system, that can be activated in different malignancies, including glioma (Spiegl-Kreinecker et al., 2002).

Glioma derived EVs may play an essential role in drug resistance through several possible mechanisms. First, cancer cells may escape intracellular drug accumulation by using EVs to export the drug. Supporting this notion, Shedden et al., investigated the encapsulation and expulsion of different anti-cancer drugs by EVs, and any correlation to drug sensitivity in different cancer models (Shedden, Xie, Chandaroy, Chang, & Rosania, 2003). Expression analysis for vesicle shedding genes in several cancer lines showed that both shedding index and GI50 (50% inhibitory drug concentrations) index for 171 compounds (drugs) were predominantly having positive relationships. Importantly, doxorubicin (Dox), a drug that blocks DNA and RNA synthesis by inhibiting topoisomerase II, extended the survival time of glioma-bearing animals and was detected in EVs, confirming the hypothesis that drugs are physically expelled into extracellular environments by EVs (Shedden et al., 2003). EVs may also regulate
the expression of genes involved in drug resistance mechanisms, including efflux pumps of the ABC family and/or enzymes essential for drug-induced DNA damage repair, or may transfer the mRNA of these genes between cells. Consistent with this hypothesis, analysis of EVs derived from GBM patients’ blood, showed that they contained abundant mRNA for MGMT and APNG (alkylpurine-DNA-N-glycosylase), key enzymes capable of repairing TMZ-induced DNA damage. The levels of these enzymes in tissues are inversely related to treatment efficacy (Shao et al., 2015). Since EV-containing mRNA is often fragmented, it is unclear whether this material can trigger chemoresistance in EV recipient cells, but horizontal transfer of other enzymes such as luciferase and Cre recombinase has been reported both in vivo and in vitro (Lai et al., 2015; Skog et al., 2008; Zomer et al., 2015).

Recently, it has been shown that EVs derived from glioma cells harbouring a PTPRZ1-MET fusion (ZM fusion), which is correlated with poor survival of GBM patients, conferred TMZ resistance to recipient GBM cells (Zeng et al., 2017). These resistant cells acquired the expression of CD133, which has previously been strongly associated with resistance of several chemotherapies (Liu et al., 2006; Zeng et al., 2017). Interestingly, CD133 can also be released in EVs from glioma cells (Redzic, Ung, & Graner, 2014). Moreover, divergent evolution of TMZ resistance in glioblastoma stem cells was reflected in their EVs (Garnier et al., 2017). The mRNA expression profiles of individual tumors derived from the same isogenic glioma stem cell line that expressed divergent and complex profiles of TMZ resistance markers, were recapitulated in the transcriptome of EVs released by these cells into the culture medium (Garnier et al., 2017). Together these data confirm that EVs released by glioma cells contribute to the resistance of chemotherapy by either expelling these drugs or through transferring
molecules that mediate chemoresistance in recipient cells. As well, EVs can be used to predict the outcome of current glioma chemotherapy.

1.3.4.5 The role of glioma derived EVs in modulating the immune response

Glioma cells secrete high levels of inhibitory cytokines, such as interleukin-10 (IL-10) and transforming growth factor beta (TGF-β), that suppress immune cells in order to evade the anti-tumor immune response, allowing their development and progression. (Qiu et al., 2011). In addition, invasive glioma cells evade immune detection by suppressing expression of antigen-presenting MHC molecules on their surfaces (Zagzag et al., 2005). Several studies have shown that glioma-derived EVs are also involved in generating an immunosuppressed microenvironment (de Vrij et al., 2015; Graner et al., 2009). EVs isolated from the blood of GBM patients contain the inhibitory cytokine TGF-β, and may be involved in suppressing immune cells (Graner et al., 2009). Furthermore, incubation of glioma-derived EVs with peripheral blood monocytes reduced their expression of the major histocompatibility complex II (MHC-II) (de Vrij et al., 2015). These EVs also appeared to skew the differentiation of peripheral blood-derived monocytes to alternatively activated M2-type macrophages, which have been found to enhance tumor growth. Incubation of these EVs with primary human microglia resulted in increased expression of membrane type 1-matrix metalloproteinase, a marker for GBM microglia and functioning as tumor-supportive factor (de Vrij et al., 2015). Another study showed that both EVs and cytokines in the serum of GBM patients could promote a M2-like monocyte response (Harshyne, Nasca, Kenyon, Andrews, & Hooper, 2016). All these findings suggest that glioma-derived EVs are involved in establishing an immunosuppressive microenvironment through interacting with immune cells to acquire characteristics that resemble the tumor-supportive phenotypes observed in GBM patients.
1.3.5 Clinical application of glioma derived EVs

1.3.5.1 Glioma derived EVs can serve as diagnostic and prognostic biomarkers

Identification of diagnostic and prognostic biomarkers for detecting patients with GBM in the early stages, defining risk groups, monitoring response to therapy, and early detection of tumor relapse, have become an interest of many scientific groups and currently are considered an integral component of clinical trials in oncology (Freidlin & Korn, 2014). The clinical importance of immunohistochemical or genetic biomarkers derived from biopsies or resected tumors is well recognized, but repeated sampling of tumor tissue is not always appropriate, particularly with brain tumors, which represent a significant medical challenge due to their anatomical location, functional impact, and biological complexity. Therefore, circulating biomarkers are crucial to avoid repeated biopsies. The 'liquid biopsy' has been implanted in clinical practice and used to monitor the progression and treatment of several cancers including breast and colorectal cancer (Diaz & Bardelli, 2014). In the future, the need for repeated biopsies of solid tumors after confirming the diagnosis from the initial resected tumor tissues, will become increasingly rare.

One of the major causes of failure in the management of GBM patients is the lack of therapeutically convenient monitoring tools. For instance, magnetic resonance imaging (MRI), which is the gold standard technique currently used for brain tumor diagnosis and detecting the tumor relapse, only detects already growing tumors with several hundreds or thousands of cells, therefore, MRI is not always useful for highly infiltrative tumors like GBM, and could lead to false-negative diagnoses (Sorensen, Batchelor, Wen, Zhang, & Jain, 2008). Another method for monitoring tumor progression is isolating and analysing circulating tumor cells. Although, circulating tumor cells are the most reflective mirror of the genetic make-up of a tumour, they
cannot reflect the heterogeneous composition that characterizes most tumors, particularly those as complex as GBM (Sottoriva et al., 2013). In addition, the existence of circulating tumor cells originating from GBM was only reported very recently by several independent groups using different methodologies, and in some cases only one cell was found per patient in a 10 ml blood sample (Macarthur et al., 2014; C. Muller et al., 2014; Sullivan et al., 2014), highlighting the need for using a comprehensive biomarker that could reflect the GBM heterogeneity and be used as a diagnostic and prognostic marker.

EVs derived from bio-fluids of GBM patients could serve as powerful biomarkers for several reasons: first, they are easy and fast to collect via non-invasive procedures as opposed to intracranial tissue biopsies. Second: their composition reflects the genetic and cellular status of the original tumor, for example, oncogenic EGFRvIII receptor was detected EVs isolated from blood of GBM patients and tumor–bearing animals (Al-Nedawi et al., 2008; Skog et al., 2008). mRNA levels of MGMT in circulating EVs from GBM patients was also shown to correspond with the identity of the parental tumor and be predictive of the current response to treatment (Shao et al., 2015), specific mutant mRNAs, such as those producing altered IDH1 were detected in EVs isolated from CSF and serum of glioma patients (W. W. Chen et al., 2013). EVs were also found to mirror changes produced in a hypoxic environment of glioma cells (Kucharzewska et al., 2013).

Another reason for considering EVs as a promising biomarker is that their half-life seems to be short in the circulation system, suggesting they could be the most accurate sampling of biomarkers, reflecting rapid and dynamic changes in the tumor cell state (Morishita et al., 2015). Moreover, isolating and analyzing EVs from different bio-fluids such as blood, CSF and urine might be combined to define the tumor heterogeneity in GBM patients. Altogether, all recent findings
discussed above hold promise that EV composition may provide a novel tool for early diagnosis and prognostic biomarkers in combination with current methods.
Chapter Two: Materials and methods

Cell culture

Brain tumor-initiating cells (BTICs) were established from surgical samples of patients diagnosed with either primary or recurrent glioblastoma within the Brain Tumor Stem Cell Core at the Arnie Charbonneau Cancer Institute (University of Calgary, Alberta, Canada) as previously described (Kelly et al., 2009; L. Wang et al., 2008). All established BTIC lines were maintained in serum-free culture medium (SFM) containing Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (Gibco BRL, Gaithersburg, MD, USA) (1:1) with 5 mM HEPES buffer (Gibco BRL, Gaithersburg, MD, USA), 0.6% glucose (Sigma-Aldrich, Oakville, ON, Canada), 3 mM sodium bicarbonate (Sigma-Aldrich, Oakville, ON, Canada), 2 mM glutamine (Gibco BRL, Gaithersburg, MD, USA), 25 µg/ml insulin (Sigma-Aldrich, Oakville, ON, Canada), 100 µg/ml transferrin (Sigma-Aldrich, Oakville, ON, Canada), 20 nM progesterone (Sigma-Aldrich, Oakville, ON, Canada), 10 µM putrescine (Sigma-Aldrich, Oakville, ON, Canada), and 30 nM selenite (Sigma-Aldrich, Oakville, ON, Canada), or in SFM supplemented with epidermal growth factor (EGF) (20 ng/ml; Peprotech, Rocky Hill, NJ, http://www.peprotech.com), fibroblast growth factor 2 (FGF2) (20 ng/ml; R&D Systems Inc., Minneapolis, http://www.rndsystems.com), and heparin sulfate (2 µg/ml; R&D Systems) at 37°C in a humidified 5% CO₂ incubator. BTIC spheres were grown until they reached a size adequate (∼100–200 µm) for passaging. Spheres were then dissociated into a single-cell suspension using Accumax, (Innovative Cell Technologies, San Diego, USA), resuspended in SFM, counted using trypan blue (Gibco BRL, Gaithersburg, MD, USA) to exclude dead cells, and plated at a density of 20,000 viable cells per ml.

The human glioma cell lines U87-MG and U251 were maintained in complete media containing DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.1 mM
non-essential amino acids, 2 mM L-glutamine and 1 mM sodium pyruvate (Gibco BRL, Gaithersburg, MD, USA) at 37°C in a humidified 5% CO2 incubator. Cells were passaged by harvesting with trypsin (Gibco BRL, Gaithersburg, MD, USA) at 80-90% confluency.

**Restriction endonuclease digestion of the PiggyBac DNA plasmid (PBSI505A shRNA)**

A PiggyBac shRNA vector (PBSI505A-1, System Bioscience) containing a human H1 polymerase-III (pol-III) promoter for shRNA expression, and human cytomegalovirus (CMV) for green fluorescent protein (GFP) and puromycin resistant gene expression was used in this study. Five µg of the PiggyBac DNA plasmid (PBSI505A shRNA) was digested overnight at 37°C in a final volume of 30 µl containing 20 units (1 µl) of the restriction endonucleases EcoRI and BamHI in 3 µl of 10x of Cut Smart restriction enzyme digestion buffer and 3 µl of 10x bovine serum albumin (BSA). The total volume of the reaction was adjusted to 30 µl using distilled water. To confirm the DNA digestion, the digested vector was electrophoresed on a 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide (EtBr) at 90 volts for 30 minutes in 1× Tris/Borate/EDTA, (TBE) buffer. The fragment of interest was then localized using a UV trans-illuminator. The digested DNA was purified using the QIAquick Gel Extraction Kit (Qiagen, Canada).

**Gel extraction and purification of DNA**

The QIAquick Gel Extraction kit was used according to the manufacturer’s recommendations to extract and purify DNA fragments smaller than 10 kb from agarose gels. DNA was mixed with gel loading dye and subjected to electrophoresis in 1% agarose containing 0.5 µg/ml EtBr. The fragment of interest was then located in the gel using a UV trans-illuminator. The DNA fragment was excised from the agarose gel with a clean, sharp scalpel and placed in a pre-weighed microcentrifuge tube that was weighed again to calculate the slice weight. Three
volumes of buffer QG (contains guanidine thiocyanate; buffer composition is proprietary, pH ≤ 7.5) were added to 1 volume of gel (100 mg ≈ 100 µl). The sample was then incubated at 50°C until the gel slice had completely dissolved. The sample was mixed by vortexing every 2-3 minutes to help the gel dissolve. The sample mixture was applied to a QIAquick spin column that was inserted into a 2 ml collection tube and then centrifuged in a bench-top microcentrifuge for 1 minute at 13,000 rpm. To remove all traces of agarose, the flow-through was discarded, 0.5 ml of buffer QG was added to the membrane and the column centrifuged for another minute at 13,000 rpm. The column was then washed by adding 0.75 ml of buffer PE and allowing it to stand for 2-5 minutes before centrifugation at 13,000 rpm for 1 minute. The flow-through was discarded and the QIAquick column centrifuged for 1 minute at 13,000 rpm to remove residual ethanol. The column was then transferred to a 1.5 ml microcentrifuge tube and DNA eluted by adding 50 µl of buffer EB (10 mM Tris.Cl, pH 8.5) to the centre of the membrane and allowing the column to stand for 1 minute. DNA was recovered by centrifugation in a bench-top microcentrifuge for 1 minute at 13,000 rpm and quantitated using a NanoDrop microvolume spectrophotometer (Life Technologies, USA).

**PiggyBac p75 shRNA generation using complementary annealed oligonucleotides**

Each shRNA insert was designed as a synthetic duplex with overhanging ends identical to those created by restriction enzyme (RE) digestion (BamHI at the 5’ and EcoRI at the 3’). The coding region for each hairpin was contained within a single oligonucleotide (upper oligo: 5’-GATCC- 21bp sense-CTCGAG-21bp antisense-TTTTTG-3’) and its complementary equivalent (lower oligo: 5’-AGCTTCAAAAA-21bp sense-CTCGAG-21bp antisense-G-3’). The sequence of all three p75NTR shRNAs was obtained from Dhharmacon (TRCN0000058153: mature antisense sequence AATTGCCATTTACTACAGTGC TRCN0000058155: mature antisense sequence
TATGAGGTCTTGTTCTGGAGG), and BLAST analysis (http://www.pubmed.gov) suggested that it had no homology with other genes. Each duplex contained a transcription initiation base, the shRNA encoding region (sense sequence, loop sequence and anti-sense sequence), and a pol-III termination signal consisting of a run of 4 'T's.

**Annealing oligonucleotides**

The oligonucleotides were purchased at the minimal synthesis and purification scales (0.05 µM scale, PAGE purification, University of Calgary DNA core service). Each oligo was re-suspended in DNase free deionized water (DDW, 20 µM). Five µl of each complementary oligonucleotide containing the shRNA sequence flanked by sequences that are compatible with the sticky ends of EcoRI and BamHI were annealed to form a double stranded oligonucleotide that was subsequently cloned into the PiggyBac transposon vector (PBSI505A). To anneal oligonucleotides, both forward and reverse oligonucleotides were resuspended in double distilled water (ddH₂O) to a concentration of 20 µM; then a mixture of 5 µl from both forward and reverse oligonucleotides were mixed with 5 µl of 10x NEB buffer 2 and 20 µl of ddH₂O. The mixture was incubated in the PCR thermocycler for 4 minutes at 95°C and then for 10 minutes at 70°C. Finally, it was cooled to room temperature over 6 hours, and the annealed oligonucleotides stored at -20°C before use.

**Ligating of annealed oligonucleotides into the PiggyBac transposon vector**

Oligonucleotides were ligated using T4 DNA Ligase (Promega) which is an ATP-dependent enzyme that catalyzes the ligation of ds.DNA fragments between the 5’-phosphate and the 3’-hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration generated by restriction enzyme digestion (Zimmerman & Pheiffer, 1983). In order to achieve the appropriate ligation between annealed oligonucleotides and the digested, purified PiggyBac
transposon vector (PBSI505A), 20 ng of the vector were mixed with 2 µl of annealed oligonucleotides, 2 µl of 10x NEB T4 DNA ligase buffer and 1 µl of NEB T4 DNA ligase. ddH₂O was added to a final volume reaction of 20 µl. Samples were incubated overnight at 16°C and then stored at -20°C before use.

**Transformation of competent E.Coli with plasmid DNA**

Transformation of competent *E. coli* with plasmid DNA was achieved using heat shock treatment. A 100 µl sample of DH5 alpha cells was mixed gently with 15 µl of the ligated PiggyBac plasmid. A negative control containing only competent cells was also included. Samples were incubated on ice for 15 minutes and then heat shocked for 90 seconds at 42°C before the addition of 100 µl of pre-warmed LB-broth to each tube. Samples were incubated with shaking at 37°C for 25 minutes and then plated onto LB-agar plates containing 100 µg/ml ampicillin and incubated, inverted, overnight at 37°C.

**Mini-preparation and purification of plasmid DNA**

A single isolated transformed colony was used to inoculate 1 ml of LB-broth containing 100 µg/ml ampicillin. The culture was incubated overnight at 37°C with shaking at 220 rpm. A glycerol stock was prepared by removing 100 µl of the culture, mixing it with 22 µl of 80% glycerol and storing the sample at -80°C. The remainder of the culture was lysed and plasmid DNA was purified using the QIAprep method.

**Plasmid DNA purification**

The QIAprep Spin Miniprep kit allows the purification of up to 20 µg of plasmid DNA from 1 to 5 ml overnight cultures of *E.coli* in LB-broth. It results in a pure plasmid DNA preparation that is free of RNA and suitable for sequencing. The manufacturer’s recommended protocol was followed and all the centrifugation steps were performed in a conventional bench-
top centrifuge. A 900 µl aliquot of an overnight culture of E.coli was centrifuged at 13,000 rpm for 3 minutes at room temperature. The supernatant was aspirated and the sample tube inverted until all culture medium had drained away. The cell pellet was resuspended in 250 µl of buffer P1 (with added RNase A solution and LyseBlue reagent) before adding 250 µl of buffer P2 (contains sodium hydroxide). The sample was mixed gently by inverting the tube 4–6 times before adding 350 µl of buffer N3 (contains guanidine hydrochloride and acetic acid) and again mixing the sample by inversion 4–6 times. The mixture was then centrifuged at 13,000 rpm for 10 minutes. The supernatant was removed and applied to the QIAprep spin column. The column was centrifuged at 13,000 rpm for 60 seconds. The flow-through was discarded and the column was washed by the addition of 0.5 ml buffer PB and centrifugation for 60 seconds at 13,000 rpm. The flow-through was discarded and the column washed a second time by the addition of 0.75 ml buffer PE (contains 100% ethanol), and centrifugation for 60 seconds. Once again, the flow-through was discarded and the column was centrifuged for an additional 1 minute at 13,000 rpm to remove residual wash buffer. The QIAprep column was then placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 30 µl of sterile deionized water was applied to the center of the QIAprep membrane. To increase the yield of DNA, the sample was left at room temperature for 1 minute before centrifugation for 1 minute at 13,000 rpm. The concentration of the recovered DNA solution was determined by measuring OD_{260} using a NanoDrop microvolume spectrophotometer.

**Screening for the shRNA insert**

To ensure that the shRNA insert was successfully ligated by the restriction enzyme digestion, 5 colonies were inculcated from each ligation into LB + 100 µg/mL ampicillin and incubated overnight at 37°C with agitation. The next day the culture was centrifuged and DNA obtained using the QIAprep Spin Miniprep kit as explained previously. Restriction digests with
XhoI, which has a restriction site in the insert only but not in the PiggyBac plasmid, and XbaI, which has a restriction site in the PiggyBac vector but not in the insert, were used to digest the insert. One µg of purified DNA was mixed with 2 µl of 10x Cut Smart buffer and 0.8 µl from each of the XhoI and XbaI restriction enzymes, and the volume made up to 20 µl using dd.H₂O. The sample was incubated at 37°C for 2 hours. The digested products were then subjected to electrophoresis in 1% agarose containing 0.5 µg/ml EtBr and two fragments, a 2kb fragment and a 5kb fragment, which were confirmed compared to the undigested DNA. The positive clones were also sequenced and blasted against the original insert sequence to confirm the sequence was fully aligned with the original sequence.

Large scale plasmid DNA purification

The QIAGEN Plasmid Maxi Kit (QIAGEN, USA) allows the purification of up to 500 µg of plasmid DNA, depending on the copy number of the plasmid. Unless otherwise stated, all centrifugations were performed in a Beckman J2-21M/E centrifuge with fixed angle rotors (JA-17). A 50 µl aliquot of glycerol stock was used to inoculate 100 ml LB-broth containing 100 µg/ml ampicillin, which was then incubated overnight at 37°C with shaking at 220 rpm. Prior to purification of the plasmid, a glycerol stock was prepared by removing 1.7 ml of the culture into a sterile 2 ml tube and centrifuged at 13,000 rpm for 2 minutes in a microcentrifuge. After removing the supernatant, the bacterial cell pellet was resuspended in 1300 µl of LB-broth, 300 µl of 80% glycerol was added and the sample was stored at -80°C. Bacterial cells were harvested from the remainder of the culture by centrifugation at 6000 rpm at 4°C for 15 minutes. The supernatant was discarded, and the cells resuspended in 10 ml of buffer P1 containing added 100 µg/ml RNase A. Ten ml of buffer P2 was then added and the sample gently mixed by inverting the tube 4–6 times. The sample was incubated at room temperature for 5 minutes, and 10 ml of pre-chilled (4°C) buffer
P3 added. The sample was mixed gently by inverting 4–6 times, incubated on ice for 20 minutes, then mixed again and centrifuged at 12,000 rpm for 30 minutes at 4°C. The supernatant containing the plasmid DNA was then transferred to a polypropylene tube and centrifuged at 13,000 rpm (JA20 rotor) for 15 minutes at 4°C. After equilibrating the QIAGEN-tip 500 by applying 10 ml QBT buffer and allowing the buffer to pass through the column by gravity flow, the supernatant containing the plasmid DNA was applied to the QIAGEN-tip and allowed to adsorb to the resin layer by gravity flow. The QIAGEN-tip was then washed twice with 30 ml of buffer QC to remove all contaminants. DNA was eluted from the resin by adding 15 ml of buffer QF, and precipitated from the eluate by adding 0.7 volumes (~10.5 ml) isopropanol. The sample was mixed and centrifuged at 11,000 rpm for 30 minutes at 4°C. The supernatant was carefully decanted, and the DNA pellet washed with 5 ml of 70% (v/v) ethanol before centrifuging the sample for 10 minutes at 9500 rpm. The supernatant was carefully decanted without disturbing the pellet. The DNA pellet was air dried for 10 minutes and then re-suspended in sterile deionized water. The quantity of DNA was then determined a NanoDrop microvolume spectrophotometer.

Integration of the PiggyBac transposon vector into target cells

The PiggyBac (PB) transposon (Systems Biosciences, USA) is a mobile genetic element that efficiently transposes between vectors and chromosomes through a "cut and paste" mechanism. When both PB transposon and the PB transposases co-transfect into target cells, the PB transposase recognizes transposon-specific inverted terminal repeat sequences (ITRs) located on both ends of the transposon vector and efficiently moves the contents from the original sites and integrates them into TTAA chromosomal sites. After verifying the sequences of shRNA that were cloned into the PB vector, BT48 and BT147 were plated in 6-well plates (one million cells per well). Each well contained a mixture of 0.5 µg of PB transposon, 0.2 µg of PB transposase
vector, 8 µl of PureFection transfection reagent and a volume of serum-free DMEM to bring the final volume up to 50 µl. The mixture was vortexed for 20 seconds and incubated at room temperature for 15 minutes to allow PureFection/ DNA complexes to form. The mixture was then added drop-wise to target cells and swirled to disperse on all target cells. The PB transposase activity terminates after 72 hours but will integrate the shRNA into target cells. After 3 days, positive integration was assessed by the number of GFP- expressing cells under fluorescent microscopy. Positive cells were recruited for puromycin selection.

**Determining the optimal puromycin concentration**

Each cell line has a unique optimal puromycin concentration; therefore, it is important to determine the optimal puromycin concentration for every cell line separately. To achieve this, three days after PB transposon transfection of BT48 and BT147, cells were treated with puromycin ranging from 1 to 5 µg/ml diluted in BTIC-serum free medium. Cells were examined each day and media changed with fresh puromycin-containing media every other day. After three days, the minimum concentration of puromycin that resulted in complete cell death of non-transfected cells was used as an optimal puromycin concentration; for both BT48 and BT147 this was 2 µg/ml.

**Ectopic expression of p75NTR in BTICs**

Stable BT73 and BT206 p75NTR overexpressing cell lines were established using the PiggyBac transposon system (Systems Biosciences, USA). p75NTR overexpressing construct was created by cloning into a modified PB530A2-GFPLuc vector with double IRES-puro. To construct PB530A2-GFPLuc p75NTR vector, p75NTR CDS with SwaI and NotI enzyme sites was amplified from pcDNA p75NTR. For the PCR, the end of each primer contained a specific restriction enzyme (SwaI: SwaIp75NTRF 5’-ATATTTAAATATGGGGGCAGGTGCCAC-3’, or NotI: p75NTRNotIF 5’-TAGCGGCGCTCACAACCGGGGATGT-3’) for ligation into the multiple
cloning sites of the PB530A2-GFPLuc vector. p75<sup>NTR</sup> cDNA with Swal and NotI enzyme sites amplified with Q5 High-Fidelity DNA polymerase (NEB) and p75 full-length plasmid was used as a template for PCR under the following conditions: 98°C 30 seconds, followed by 35 cycles of 98°C for 10 seconds, 61°C for 20 seconds, and 72°C for 1 minute 45 seconds, with a final extension at 72°C for 2 minutes. PCR product was purified by agarose gel electrophoresis and gel extraction (QIAquick gel extraction kit). p75 cDNA (with Swal and NotI) PCR band was inserted into a TA vector (TOPO cloning kit, Invitrogen) by ligation and then transformed into E.coli XL10-Gold strain for DNA work. Screening for positive colonies that contained p75<sup>NTR</sup> cDNA was then performed followed by colony PCR with p75<sup>NTR</sup> specific primers (Hp75<sup>NTR</sup>F 5’-CGTATTCCGACGAGCCAAACC-3’, Hp75<sup>NTR</sup>R 5’-CCACAAGGGCCACAAACCACAGC-3’). The plasmids were extracted from positive colonies using the miniprep extraction kit. After cloning into an intermediate vector, the colonies were cloned into PB530A2-GFPLuc vector with the aid of Swal/ NotI enzymes. p75<sup>NTR</sup> TA plasmid and PB530A2-GFPLuc vector were digested with Swal and NotI and subjected to agarose gel electrophoresis, and then extracted for ligation using a gel extraction kit. Rapid DNA ligation kit (Roche, Canada) was used to ligate the p75<sup>NTR</sup> insert and PB530A2-GFPLuc vector. 100 ng of linearized PB530A2-GFPLuc vector were mixed with 50 ng of insert (total volume 10 µl) in 10 µl of 2x T4 ligase buffer and 1 µl of T4 ligase and then incubated at room temperature overnight. The PiggyBac vector containing p75<sup>NTR</sup> was used to stably transfect BT73 and BT206 cells as described previously.

**Western blotting**

Cells were lysed with RIPA buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% (v/v) 1% Nonidet P-40, protease inhibitor cocktail (Roche, Indianapolis, IN, USA), phosphatase inhibitor (Sigma, USA) and protein was quantified with a bicinchoninic acid (BCA) assay (Pierce
Biotechnology, Rockford, IL, USA). Proteins were diluted with 2x Laemmli sample buffer to the desired concentrations and boiled at 95°C for 5 minutes. Proteins were then resolved on 10% SDS-PAGE (polyacrylamide gel electrophoresis) gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Non-specific binding to the membranes was blocked by incubating the membranes in 5% milk in TBST [Tris-buffered saline (20 mM Tris pH 8.0, 137 mM NaCl) containing 0.2% Tween-20]. The membranes were then incubated in 5% milk in TBST containing the appropriate primary antibody: rabbit polyclonal anti-human p75<sub>NTR</sub> (dilution 1:1000, Promega, Cat: G323A), mouse monoclonal anti flotillin-1 (dilution 1:500, BD Transduction Laboratory, Cat: 610821), rabbit polyclonal anti-human TSG 101 (dilution 1:1000, Abcam, Cat: ab30871), rabbit monoclonal anti-GFAP (dilution 1:1000, Abcam, Cat: ab68428), mouse monoclonal anti-human Alix (dilution 1:1000, Cell Signaling, Cat: 2171S), rabbit polyclonal anti-Olig2 (dilution 1:1000, Millipore, Cat: AB9610), mouse monoclonal anti-actin (dilution 1:2000, Millipore, Cat: MAB1501), and mouse monoclonal anti-SOX2 (dilution 1:500, R and D system, Cat: MAB2018) for either 1 hour at room temperature or overnight at 4°C. Following thorough washing with TBST four times (five minutes each), membranes were incubated in 5% milk with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody: goat anti-mouse dilution 1:2000 or goat anti-rabbit 1:5000 (SantaCruz Biotechnology, Santa Cruz, CA, USA) for one hour at room temperature. Membranes were washed again with TBST four times (five minutes each) and then incubated with enhanced chemiluminescence (PerkinElmer, Cat: ORT2755) and visualized using ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).
Sphere-forming assay

BTIC sphere-forming assays were performed under neural stem cell conditions as described previously (Kelly et al., 2009; L. Wang et al., 2008). Actively growing BTICs were harvested by dissociating low passage number BTIC spheres into a single-cell suspension using Accumax (Innovative Cell Technologies, San Diego, USA). Viable cells were quantified using trypan blue exclusion solution (Invitrogen, USA) and 2000 viable cells were placed into 96-well plates in serum free medium supplemented with EGF (20 ng/ml; Peprotech, Rocky Hill, NJ, http://www.peprotech.com), FGF2 (20 ng/ml; R&D Systems Inc., Minneapolis, http://www.rndsystems.com), and heparin sulfate (2 µg/ml; R&D Systems) and incubated at 37°C in a humidified 5% CO₂ incubator for 7 days. Spheres >10 µm were counted and photographed using the IN Cell Analyzer 6000 cell imaging system (GE Healthcare Life Sciences, USA).

Cell cycle analysis

Actively growing BTICs were harvested by dissociating low passage number GBM spheres into a single-cell suspension using Accumax (Innovative Cell Technologies, San Diego, USA). All cells were washed and resuspended in 1 mL of PBS. One ml of cold absolute ethanol was added to the cells and they were stored at -20°C for 20 minutes to one week. The cells were then washed 2 times with cold PBS and treated with 250 µl of 1 mg/ml RNase A (Sigma-Aldrich, Oakville, ON, Canada) in PBS for 30 minutes at room temperature. Propidium iodide (Sigma-Aldrich, 250 µl of a 50 µg/ml solution in PBS) was then added and the cells incubated in the dark for 45 minutes. The intensity of the propidium iodide staining was then analyzed on a FACScan flow cytometer (Becton and Dickinson Company, Oakville, ON, Canada).
Symmetry and asymmetry cells divisions

To study symmetry and asymmetry cell divisions, BTIC single cells were plated at low density (1000 cells/coverslip) on glass coverslips coated with a thin layer of laminin (10µg/ml) for 24–30 hours. Cells were fixed with 4% paraformaldehyde, permeabilized and stained as described previously. Primary rabbit polyclonal anti-Numb antibody (1:500 dilution, Abcam Ca: AB14140) and secondary goat anti-rabbit antibody conjugated to Alexa Fluor 568 (1:500 dilution) were used in this analysis. Symmetry and asymmetry cell divisions were evaluated based on Numb positive staining. Results were quantified from at least 50 cell couples per sample.

AlamarBlue Assay

Actively growing BTICs were harvested by dissociating low passage number BTIC spheres into a single-cell suspension using Accumax (Innovative Cell Technologies, San Diego, USA). Viable cells were quantified using trypan blue exclusion solution (Invitrogen, USA) and 2000 viable cells were placed into 96-well plates (n=6) in 200 µl of serum free medium supplemented with EGF (20 ng/ml; Peprotech, Rocky Hill, NJ, http://www.peprotech.com), FGF2 (20 ng/ml; R&D Systems Inc., Minneapolis, http://www.rndsystems.com), and heparin sulfate (2 µg/ml; R&D Systems) and incubated at 37°C in a humidified 5% CO₂ incubator for 7 days. On day 7, 20 µl of AlamarBlue reagent (Life Technologies, USA) was added to each well and fluorescence measured after 6 hours. Data presented in graphs based on the fluorescent intensity.

Flow cytometric analysis of p75NTR, CD15 and CD133 expression

Actively growing BTICs were harvested by dissociating low passage number BTIC spheres into a single-cell suspension using Accumax (Innovative Cell Technologies, San Diego, USA). Viable cells were quantified using trypan blue exclusion solution (Invitrogen) and 2000 viable cells were used for flow cytometric analysis. Single cells were resuspended in PBS with 1% bovine
serum albumin (BSA) and incubated with primary antibodies for 1h at 4°C in the dark. Primary antibodies used included: anti-human p75NTR-PE (1:10 dilution, Exbio, Ca: IP-642-T100), anti CD133-APC (1:10 dilution, Miltenyi Biotec, Ca: 293C3) and anti-CD15-PE (1:10 dilution, Beckman Coulter, Ca: IM1954U). All flow cytometric analyses were performed with an ATTUNE flowcytometer (Life Technologies, USA). Gates were placed based on isotype controls. Analysis of all flow cytometry data was performed with FlowJo (Treestar, USA).

**Differentiation assay**

Actively growing BTICs were harvested by dissociating low passage number BTIC spheres into a single-cell suspension using Accumax (Innovative Cell Technologies, San Diego, USA). Viable cells were quantified using trypan blue exclusion solution (Invitrogen) and 5000 viable cells were used for differentiation assays. Cell were plated on coverslips (5000 cell/cover) in neural stem cell medium supplemented with 2% FBS. Cells were maintained in this medium for 7 days and then processed for immunofluorescence staining.

**Transwell invasion assay**

One ml of bovine collagen standard solution, type I (3 mg/ml) (PureCol, Advanced BioMatrix, CA, USA) was diluted in 9 ml of 10x DMEM and used to coat transwell inserts (8 µm pore size; Corning Costar, Corning, NY, USA). Fifty µl of collagen were incubated in each insert for 5 minutes and then removed. The lower chamber of the insert was also covered with a thin layer of collagen. The inserts were incubated in a sealed 24-well tissue culture plate overnight at 4°C. The coated transwell inserts were placed into the wells of a 24-well tissue culture plate containing 500 µl of media supplemented with 10% heat-inactivated FBS (lower chamber). Cells (1x10^6) were seeded into the upper chamber, in 200 µl of the same media contained in the bottom of the well, and incubated for 6 hours at 37°C in a 5% CO2 incubator. The media was then removed
from the chambers and cells scraped off the top of the membrane using a PBS-soaked cotton-tipped swab. Cells were fixed to the bottom of the chamber with 1% crystal violet in 95% ethanol for 1 minute. Invasion was quantified by counting the stained cells that were adherent to the lower side of the membranes in five random fields of view (at 10x magnification).

**Animals**

Six- to eight-week-old female severe combined immunodeficient (SCID) mice were purchased from Charles River Canada (Saint-Constant, QC, Canada). The animals were housed in groups of five in soft bedding cages and maintained on a 12-hour light/dark schedule at 22°C ± 1 °C and a relative humidity of 50% ± 5%. Food and water were available as desired. All procedures were reviewed and approved by the University of Calgary Animal Care Committee. All animal work procedures were in accordance with the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals.

**Intracranial glioma model**

Actively growing BTICs were harvested by dissociating low passage number GBM spheres into a single-cell suspension using Accumax (Innovative Cell Technologies, San Diego, USA). All cells were resuspended in SFM and cells counted using trypan blue exclusion to identify viable cells. Either 10,000 or 100,000 viable cells were resuspended in 2 µl of sterile 1x phosphate-buffered saline (PBS) for stereotactic implantation. Cells were implanted intracerebrally into the right striatum of the SCID mice described above (1 x 10^5 cell/mouse for BT48 and BT147 and 1x10^4 cells/mouse for BT73M and BT206) at a depth of 3 mm through a scalp incision and a 0.5-mm burr hole made 1.5–2 mm right of the midline and 0.5–1 mm posterior to the coronal suture. All mice were anaesthetized by intraperitoneal administration of ketamine (85 mg/kg) plus
xylazine (15 mg/kg) (MTC Pharmaceuticals, Cambridge ON, Canada). The stereotactic injection used a 5-µl syringe (Hamilton Co., Reno, NV) with a 30-gauge needle mounted on a Kopf stereotactic apparatus (Kopf Instruments, Tujanga, CA). After withdrawal of the needle, the incision was sutured. Mice were monitored weekly and sacrificed at specific time points (BT48 at 3 months post-injection; BT147, BT73M and BT206 at 6 weeks post-injection) or when they lost 20% of their body weight or had difficulty ambulating, feeding, or grooming. The brains were extracted and fixed in 10% formalin and paraffin-embedded. Whole brain tissue sections from all mice in each group were examined by immunohistochemistry. All analyses included at least six animals per experimental group from three independent experiments.

**Immunohistochemistry**

Frozen sections of the brain tissue were air-dried at room temperature, fixed with cold acetone and rinsed with PBS. Paraffin sections were dewaxed and rehydrated using a xylene/ethanol series followed by rinsing with PBS. Endogenous peroxidases were inactivated with 0.075% H$_2$O$_2$/methanol, and nonspecific binding blocked with Rodent Block M (Biocare Medical, Concord, CA, USA). Sections were incubated with 1:500 diluted rabbit polyclonal anti-p75$^{NTR}$ antibody (Promega), 1:500 diluted mouse monoclonal anti-human nucleolin (Abcam, Cat: ab13541) 1:1000 diluted rabbit monoclonal anti-GFAP (Abcam, Cat: ab68428), 1:1000 diluted rabbit polyclonal anti-Olig2 (Millipore, Cat: AB9610), 1:500 diluted rabbit polyclonal anti-Iba1 (Dako, Cat: LNK5648), 1:50 diluted rat monoclonal anti-Brdu (Abcam, Cat: ab6326) and 1:500 diluted rabbit polyclonal anti-Nestin (R and D system, Cat: MAB1259). Brain sections were incubated with the primary antibodies either overnight at 4°C or for 1 hour at room temperature. Brain sections were washed with Tris buffered saline (TBS) three times (2 minutes each), then, the appropriate biotinylated secondary antibody (Vector Laboratories, Burlington, ON, Canada)
was applied. Avidin-biotin peroxidase complexes were then formed using the VECTASTAIN Elite ABC kit (Vector Laboratories) and detected by addition of SIGMAFAST™ DAB (3,3’-diaminobenzidine tetrahydrochloride, Sigma-Aldrich), which was converted to a brown reaction product by the peroxidase. Hematoxylin (for paraffin sections) and toluidine blue (for frozen sections) were used as nuclear counterstains. Sections were then dehydrated in an ethanol/xylene series and mounted with Entellan (Electron Microscopy Sciences).

**Immunofluorescence staining**

BTICs grown on laminin (L202; Sigma) coated coverslips were fixed in 4% paraformaldehyde and permeabilized with addition of 0.1% Triton-X100 prior to immunostaining. Cells were treated with primary rabbit polyclonal anti-p75NTR antibody (Promega, Cat G323A), mouse monoclonal anti-Numb (Abcam, Cat: AB1410), rabbit monoclonal anti-GFAP (Millipore, Cat: AB68428) and secondary antibody conjugated to Alexa Fluor 568 (Invitrogen Molecular Probes) diluted 1:500 in PBS/EDTA were sequentially applied on samples for 30 minutes on ice in the dark. Cell were washed three times (3 minutes each) and cell nuclei were counterstained with 2 µg/ml of DAPI (4’,6-diamidino-2-phenylindole) diluted in H2O for 5 minutes in the dark. Coverslips were mounted onto the glass slides visualized using either the fluorescent microscope (Leica Microsystems Inc., Richmond Hill, ON, Canada) or the IN Cell Analyzer 6000 cell imaging system (GE Healthcare Life Sciences, USA).

For whole brain tissue sections, paraffin-embedded tissues were deparaffinized with xylene, rehydrated through an ethanol gradient (100%, 100%, 95%, 70%) for 2 minutes each and then washed in ddH2O for 2 minutes. Antigen retrieval was achieved by a heat-induced epitope retrieval method. Briefly, slides were incubated in 1x rodent decloaker, the antigen retrieval buffer (Cat: RD913M, Biocare Medical, CA, USA) and heated in a pressure cooker for 45 minutes at
95°C and then for 6 minutes at 121°C. Samples were cooled to room temperature and washed 3 times in 1X TBS wash buffer for 2 minutes each. Samples were then blocked in Rodent block M (Cat: RBM961L, Biocare Medical, CA, USA) at room temperature for 30 minutes and washed 3 times in 1X TBS wash buffer. Samples were incubated with the appropriate primary antibodies diluted in Signal Stain Antibody Diluent (Cat: 8112L, Cell Signaling Technology, Danvers, MA, USA) including: 1:500 diluted rabbit polyclonal anti-p75NTR antibody (Promega), 1:500 diluted mouse monoclonal anti-human nucleolin (Abcam, Cat: ab13541) 1:1000 diluted rabbit monoclonal anti-GFAP (Abcam, Cat: ab68428), 1:1000 diluted rabbit polyclonal anti-Olig2 (Millipore, Cat: AB9610), 1:500 diluted rabbit polyclonal anti-Iba1 (Dako, Cat: LNK5648), 1:50 diluted rat monoclonal anti-Brdu (Abcam, Cat: ab6326) and 1:500 diluted rabbit polyclonal anti-Nestin (R and D system, Cat: MAB1259). All antibodies were diluted in the antibody diluent (Biocare Medical, USA). Brain sections were incubated with the primary antibodies either overnight at 4°C or for 1 hour at room temperature. Brain sections were washed with TBS three times for 2 minutes each, then the appropriate secondary antibody conjugated to Alexa Fluor 568 or Alexa Fluor 488 (dilution 1:500) (Invitrogen Molecular Probes) was applied for one hour at room temperature. Brain sections were washed with TBS three times for 2 minutes each, and slides were mounted and visualized using either the fluorescent microscope (Leica Microsystems Inc., Richmond Hill, ON, Canada) or the IN Cell Analyzer 6000 cell imaging system (GE Healthcare Life Sciences).

**Opal multiplex immunohistochemistry assay**

A major advantage of using multicolor immunohistochemistry (IHC) is the ability to visualize biology within biological samples, thereby expanding our understanding of the complex cellular interactions that is not achievable by other methods. Opal multiplex IHC follows standard
IHC by starting with deparaffinization and rehydration of tissue samples followed by heat-based antigen retrieval, a blocking step, incubation with an unlabelled primary antibody, and finally the addition of anti-species-HRP conjugate and Opal fluorescent detection substrate. For detecting another target, an antigen retrieval step must be done to remove unspecific antibody binding from the previous step and to clear the tissue for the next epitope detection; then another round of staining can be performed. Once all targets have been labelled, tissues can be counterstained with DAPI and mounted to image using the IN Cell Analyzer 6000 cell imaging system (GE Healthcare Life Sciences, USA).

**Microarray**

RNA was extracted from 500,000 cells using mirVana miRNA Isolation Kit (Ambion, Austin, USA) according to the manufacturer’s protocol. Genomic-free RNA was purified with RNaseasy PlusMicro Kit (Qiagen, Valencia, USA) and RNA ‘quality of integrity’ number (RIN) was measured using the Agilent RNA 6000 NanoChip on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). RNA quantity was measured using a NanoDrop 1000 (NanoDrop Technologies, Inc, Wilmington, USA) and 100 ng of RNA with a RIN higher than 9 was labeled with 3' IVT Express Kit (Ambion) and hybridized to Affymetrix GeneChip Human PrimeView Arrays at 45°C for 16 hours. Arrays were stained using Affymetrix GeneChip Fluidics_450 following the manufacturer’s protocol and scanned using the Affymetrix GeneChip Scanner 3000 7G System. The raw data sets for array comparisons have been deposited in the Gene Expression Omnibus website: http://www.ncbi.nlm.nih.gov/geo/(accession number: GSE76146). Ten Microarray Data Analysis Affymetrix GeneChip array data files were generated using GeneChip® Command Console® Software (AGCC).
Preparation of conditioned medium for extracellular vesicle isolation

To prepare conditioned medium (CM), 2 x 10⁷ U87 or U251 glioma cells or BTICs were washed twice with cold PBS then incubated in 15 ml of serum free DMEM at 37°C in a humidified 5% CO₂ incubator for 48 hours. CM was then collected and centrifuged for 10 min at 200 x g and 4°C to remove any cell contamination. Cell counting was performed with trypan blue staining (Life Technologies, Carlsbad, California, USA) to assess cell viability. To remove any apoptotic bodies or possible extra cell debris, CM was filtered through 0.22 µm low protein binding polyethersulfone (PES) membranes (Thermo Fisher Scientific, Waltham, Massachusetts, USA). CM was then used to isolate extracellular vesicles (EVs) using differential ultracentrifugation, optiprep density gradient or a synthetic peptide with specific affinity for heat shock proteins (New England Peptide Inc, Massachusetts, USA).

Isolation of extracellular vesicles

EVs were isolated from conditioned medium, serum of glioblastoma patients and serum of brain tumour-bearing mice using one of the following methods.

Differential ultracentrifugation

EVs were isolated from CM using differential ultracentrifugation method as described previously (Thery, Amigorena, Raposo, & Clayton, 2006). Briefly, CM of cells grown for 48 hours were collected and centrifuged at 200 x g for 10 minutes to remove cells. CM was sequentially centrifuged at 2000 x g for 10 minutes and 10,000 x g for 30 minutes, and then filtered through 0.22 µm low protein binding PES membranes and centrifuged at 100,000 x g for 90 min. The pellet (P100) containing EVs was washed once with 10 ml of cold PBS and centrifuged again at 100,000 x g for 90 min. P100 was resuspended in buffers suitable for the downstream analysis. All centrifugation was performed at 4°C.
Optiprep density gradient centrifugation

To isolate EVs using Optiprep density gradients, 15 ml of filtered CM was concentrated into 1 ml using Amicon Ultra-15 Centrifugal Filter Units (Millipore, Massachusetts, USA). Optiprep (Axis-Shield, Oslo, Norway) density gradients were prepared as previously described (Vergauwen et al., 2017) with some modifications. Briefly, different concentrations of iodixanol (5%, 10%, 20% and 40%) were prepared by mixing appropriate amounts of homogenization buffer [0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCL, (pH 7.4)], iodixanol working solution buffer [0.25 M sucrose, 6 mM EDTA, 60 mM Tris-HCl, (pH 7.4)] and OptiPrep™ stock solution (60% (w/v) aqueous iodixanol solution). A discontinuous iodixanol gradient was prepared by layering 2.5 ml of 40%, 20%, 10% and 5% iodixanol in an 11 ml open top ultra-clear tube (Beckman Coulter, Fullerton, California, USA). One ml of concentrated CM was layered on top of the gradient, followed by 18 hours ultracentrifugation at 100,000 x g and 4°C using SW 41 Ti rotor (Beckman Coulter, Fullerton, California, USA). To detect the fractions that were enriched with EVs, 11 gradient fractions of 1 ml were collected from the top of each gradient and every fraction was transferred into a new ultra-clear tube, diluted with 10 ml of cold PBS and centrifuged at 100,000 g and 4°C for 3 hours. The pelleted EVs were resuspended in the appropriate buffer required for the downstream experiments.

EV isolation using Vn peptides

Venceremín (Vn) peptides were used as described previously (Ghosh et al., 2014). Briefly, 1 ml of the filtered CM was incubated with 50 g/ml Vn peptide at room temperature with rotation. The samples were then centrifuged at 10,000 x g at room temperature for 7 minutes. Semi-translucent precipitates were collected and washed three times with cold PBS, then resuspended in the appropriate buffer for the downstream analysis.
For isolating EVs from serum of GBM patients or brain tumor bearing-mice, serum was diluted 10 times with PBS. The samples were then subjected to cleaning by centrifugation at 17,000 x g for 15 minutes at 4°C. Samples were filtered through 0.22 µm low protein binding PES membranes. The samples were incubated with 50 g/ml of Vn peptide overnight at 4°C with rotation, followed by centrifugation at 17,000 x g at 4°C for 15 minutes and three washes with PBS. The isolated EVs were then resuspended in the appropriate buffer for the downstream analysis.

**Extracellular vesicle analysis using scanning electron microscopy**

In order to visualize EVs budding from glioma cells, cells were plated on glass coverslips (5000 cells/coverslip) and incubated in DMEM supplemented with 10% FBS for 48 hours. Cells were then washed twice with cold PBS and incubated in serum-free medium for 24 hours. The cells were then washed twice with cold PBS and fixed with 2% glutaraldehyde overnight at 4°C. Following fixation, cells were washed twice with cold PBS for 30 minutes each. Cells were then dehydrated in an ascending series of ethanol concentrations (25, 40, 60, 75, 85, 90, 100%) at 4°C for 10 minutes each, and incubated in an ascending series of hexamethyldisilazane (HDMS) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) diluted in ethanol (25, 50, 75, 100%) at room temperature for 10 minutes each. HDMS was removed and cells were dried by incubating them at room temperature overnight. Cells were then imaged using scanning electron microscopy at the imaging facility of the University of Calgary.

**Extracellular vesicle analysis using transmission electron microscopy**

In order to assess the morphology and the purity of EVs using TEM, 5 µl of EV-containing PBS were adsorbed for 20 minutes on copper grids covered with former film and stabilized by carbon. Grids were washed twice with 100 µl drops of PBS for 5 minutes each and EVs were then
fixed in 50 µl of 1% glutaraldehyde for 5 minutes. Following fixation, EVs were washed 8 times in 100 µl PBS to remove excess glutaraldehyde. EVs were then contrasted in a solution of uranyl oxalate (pH 7) for 5 minutes and embedded in a mixture of 4% uranyl acetate and 2% methyl cellulose in a ratio of 100 µl/900 µl, respectively for 10 minutes on ice. Grids were air dried overnight at room temperature and EVs were imaged using transmission electron microscopy at the imaging facility of the University of Calgary.

**Fluorescence labelling of extracellular vesicles**

EVs were fluorescently labelled with either PKH2 (Sigma-Aldrich, Oakville, ON, Canada) or Cell tracker labelling dye (Life Technologies, USA) according to the manufacturer’s instructions. Briefly, 10 µg of isolated EVs were incubated with freshly prepared PKH2 (500 µl) for 5 minutes at room temperature. Labelled EVs were mixed with 500 µl of 1% BSA and then pelleted at 110,000 x g using a tabletop ultracentrifuge (Beckman Coulter, Fullerton, California, USA) for 70 minutes at 4°C. The pelleted EVs were washed in 1 ml of cold PBS and pelleted again at 110,000 x g for 70 minutes at 4°C. The pelleted EVs were recruited for a final wash step in 1 ml of cold PBS to remove excess dye and pelleted again at 110,000 x g for 70 minutes at 4°C. The pelleted EVs were re-suspended in 50 µl of PBS. As a negative control and to ensure lack of fluorescent micro particle contaminants, a control tube containing dye without EVs was used for the same steps and was used as a negative control in EVs uptake experiments.

**Extracellular vesicles uptake**

For fluorescent microscopy analysis of EV uptake, U87 glioma cells were seeded at 1000 cells/cover slip in a 24-well plate. After 24 hours, cells were washed two times with PBS and incubated with fluorescently labelled EVs (5 µg) in serum-free medium for 24 hours. Cells were
washed with PBS, then with acid wash buffer (0.5 M NaCl, 0.2 M acetic acid) to remove membrane-bound EVs and washed once again with PBS. Cells were then fixated with 4% paraformaldehyde in PBS at room temperature for 20 minutes. After fixation, cells were stained with Alexa Fluor 568 phalloidin, which is a high-affinity filamentous actin (F-actin) probe. Cells were washed three times (3 minutes each) and cell nuclei were counterstained with 2 µg/mL of DAPI (4',6-diamidino-2-phenylindole) diluted in H₂O for 5 minutes in the dark. Coverslips were mounted onto the glass slides and visualized using either the fluorescent microscope (Leica Microsystems Inc., Richmond Hill, ON, Canada) or the IN Cell Analyzer 6000 cell imaging system (GE Healthcare Life Sciences, USA).

**Statistical analysis**

All data reported for *in vitro* experiments are representative of at least three independent replicates. Statistical analysis was performed using GraphPad Prism (version 7; GraphPad Software, Inc.). Survival curves were generated using the Kaplan–Meier method. The log-rank test was used to compare the distribution of survival times. A *P* value of ≤ 0.05 was considered statistically significant.
Chapter Three: Cell-autonomous mechanisms of neurotrophin receptor p75\textsuperscript{NTR} in glioblastoma progression

3.1 Introduction

3.1.1 Rationale

Glioblastoma (GBM) is a highly aggressive brain tumor with a complex biology, and despite the highly intensive research on GBM, mechanisms by which it arises and progresses are still not well-understood. GBM remains mostly incurable with a very poor prognosis. GBM tumors have different hallmarks that include: sustaining proliferative signaling, evading growth suppressors, activating invasion mechanisms, enabling replicative immortality, inducing angiogenesis, resisting cell death, evading immune destruction and reprogramming cellular energetics (J. Chen, McKay, et al., 2012). Glioma stem-like cells or brain tumor initiating cells (BTICs) isolated from GBM tumors may be the reservoir of gliomagenesis, glioma progression and glioma relapse (S. K. Singh et al., 2004). BTICs share several characteristics with normal neural stem cells including the expression of various neural stem cell markers such as CD133, CD15, Nestin and SOX2 (Gangemi et al., 2009; Mangiola et al., 2007; S. K. Singh et al., 2004), self-renewal capacity, and neuronal multilineage differentiation. BTICs can form highly invasive tumors when transplanted into immunodeficient mice, and these tumors resemble human GBM tumors genetically and phenotypically (Galli et al., 2004; S. K. Singh et al., 2004). In addition, BTICs are relatively resistant to both radiotherapy and chemotherapy (Bao et al., 2006; J. Chen, Li, et al., 2012), suggesting that they play a major role in the tumor progression, resistance to treatments and tumor relapse.

As mentioned in the general Introduction our lab used a novel unbiased \textit{in vivo} serial transplantation strategy to discover the genes that are essential for invasive behaviour of GBM and
found that the $p75^{NTR}$ gene was one of the highly upregulated genes in invasive cells compared to non-invasive cells (A. L. Johnston et al., 2007). Through a series of biochemical, functional and clinical studies, $p75^{NTR}$ was found to dramatically enhance invasion and migration of genetically distinct glioma cells and to frequently have a robust expression in highly invasive GBM patient tissues. In a follow-up study, our laboratory demonstrated that regulated intramembrane proteolysis of $p75^{NTR}$ by $\gamma$ secretase is an essential biochemical process for glioma invasion, and the inhibition of this process by generating genetically $p75^{NTR}$ cleavage-resistant mutants or by using clinically relevant $\gamma$ secretase inhibitors significantly decreased the highly invasive behaviour of genetically distinct GBM cells (L. Wang et al., 2008).

Upon neurotrophin binding, $p75^{NTR}$ signals alone or in combination with other receptors to regulate a wide range of biological processes, which are highly cell type and cell context specific. These processes include cell survival and cell death, myelin formation and neurite outgrowth (Alshehri et al., 2017). In addition, $p75^{NTR}$ regulates proliferation and differentiation of both neuronal and non-neuronal cells (Cattaneo & McKay, 1990; Seidl, Erck, & Buchberger, 1998). Cattaneo & McKay (1990) found that neural precursor cells that express the intermediate filament protein, nestin, proliferate in the presence of nerve growth factor (NGF), which binds the $p75^{NTR}$, and on withdrawal of NGF, the proliferative cells differentiate into neurons. Importantly, $p75^{NTR}$ was expressed by a small population of cells within the stem cell niche of the rat subventricular zone (SVZ) and these cells are responsible for neuron production in both newborn and adult animals (K. M. Young, T. D. Merson, A. Sotthibundhu, E. J. Coulson, & P. F. Bartlett, 2007). In the context of cancer, $p75^{NTR}$ is expressed by cancer stem cells in several cancers including melanoma (A. D. Boiko et al., 2010), squamous cell carcinoma (S. D. Huang et al., 2009), breast cancer (Kim et al., 2012), and hypopharyngeal cancer (Imai et al., 2013). In glioma, our laboratory
previously showed that p75NTR is the main regulator of glioma invasion and was expressed on a subset of patient-derived BTICs and in highly invasive GBM patient tissues (Ahn et al., 2016; Alshehri et al., 2017; A. L. Johnston et al., 2007; L. Wang et al., 2008); however, the role of p75NTR in the signaling pathways and progression of glioma, and the biology of glioma stem cells is still not fully identified. Here, we studied the cell-autonomous roles of p75NTR using loss and gain of function studies in different BTICs to identify the requirement of p75NTR in sphere-forming capacity, proliferation and differentiation of BTICs both in vitro and in vivo.

3.1.2 Hypothesis
Our hypothesis is that p75NTR contributes to glioma progression by regulating sphere-forming capacity, proliferation, invasion and differentiation of patient derived-BTICs.

3.1.3 Objectives
To address this hypothesis, two main objectives were outlined. The first was to determine whether loss of function of p75NTR in GBM patient-derived BTICs affects their sphere-forming capacity, proliferation and differentiation in vitro and reduces their tumorigenicity in vivo. The second objective was to determine if the gain of function of p75NTR in GBM patient-derived BTICs increases their stemness and thereby their tumorigenicity.

3.2 Results
3.2.1 BTICs express variable levels of p75NTR

p75NTR has been implicated in regulating cell proliferation and survival in various cell types (M. V. Chao, 2003). In the majority of malignant tumors including melanoma, breast cancer,
squamous cells carcinoma, head and neck cancers, p75<sup>NTR</sup> signaling has been found to contribute to the tumor progression (reviewed in (Tomellini et al., 2014). In glioma, p75<sup>NTR</sup> was found to express on a subset of GBM patient–derived BTICs and correlated with their invasive behavior (Ahn et al., 2016; Alshehri et al., 2017; A. L. Johnston et al., 2007; Tomellini et al., 2014; L. Wang et al., 2008). To establish the rationale for studying loss of function of p75<sup>NTR</sup> in GBM patient–derived BTICs, we examined the protein level of p75<sup>NTR</sup> by western blot in several GBM patient–derived BTICs (BT94, BT245, BT206, BT241, BT248-XY, BT191, BT69, BT48, and BT147) (Figure 3.1.A). These BTICs have been previously characterized for their stem cells properties in vitro and in vivo (Ahn et al., 2016; Cusulin et al., 2015; Kelly et al., 2009; Lun et al., 2016). Western blot analysis showed that p75<sup>NTR</sup> was variably expressed in the tested BTIC line, with BT48 and BT147 displaying the highest p75<sup>NTR</sup> expression (Figure 3.1.A). Interestingly, the genomic DNA sequencing of these cells revealed that they harbored mutations that have been linked to GBM progression. While BT48, which was isolated from a primary adult-GBM tumor, was found to have mutations in EGFR and PTEN but was normal for both p53 and IDH1, BT147, which was isolated from a recurrent adult-GBM tumor, was found to harbor the mutated EGFR isoform, EGFRvIII, and have mutations in both PTEN and p53 but was normal for IDH1 (Figure 3.1.B). Although neurosphere cultures of BTICs is the gold standard method of maintaining these cells in vitro, the cultures do not recapitulate the tumor microenvironment, and as the spheres expand, cellular plasticity, loss of expression of stem cell markers and internal cellular heterogeneity increase, most likely due to diffusion limitations of growth factors, oxygen, and metabolic factors (Lathia, Mack, Mulkearns-Hubert, Valentim, & Rich, 2015). Thus, we sought to determine if the screened BTICs have similar levels of p75<sup>NTR</sup> expression in the tumor microenvironment compared to that we observed in vitro before proceeding to p75<sup>NTR</sup> loss and
gain of function studies. Using intracranial murine xenograft models, all selected BTICs were implanted into the brains of severe combined immunodeficiency (SCID) mice. All tested BTIC lines were tumorigenic and expressed variable levels of p75NTR \textit{in vivo}. Similar to our \textit{in vitro} observations, double immunofluorescence staining of brain sections from all tested BTIC lines showed that BT48 and BT147 lines expressed higher levels of p75NTR \textit{in vivo} compared to the rest of the tested BTIC lines (Figure 3.1.C). In addition, RNA-seq analysis showed that BT147 and BT48 have the highest transcript reads of p75NTR compared to the other BTIC xenografts (Figure 3.1.D). Collectively, these results suggest that BTICs have a variable expression of p75NTR both at the mRNA and protein levels. To assess the functional significance of p75NTR in the biology of GBM patient-derived BTICs, and because both BT48 and BT147 are genetically distinct and have high p75NTR protein levels, these lines were selected for p75NTR loss of function studies. To assess the role of p75NTR in glioma progression, both BT48 and BT147 were stably transfected using the PiggyBac transposon system that either contains p75NTR shRNA or control shRNA. Western blot analysis showed that p75NTR expression was dramatically reduced in both BT48 and BT147 transfected with p75NTR shRNA (p75NTR KD) compared with the control shRNA in both BTIC lines (Figure 3.2.A). These results were confirmed with the immunofluorescent staining of p75NTR in both BT48 and BT147 p75NTR KD and their control counterparts (Figure 3.2.B). Flow cytometry analysis of p75NTR expression showed that p75NTR expressing cells represent \textasciitilde28\% of BT48, and upon p75NTR knockdown, this population fell to \textasciitilde11\% in BT48p75NTR KD cells. BT147 has 50\% of p75NTR expressing cells, which upon p75NTR knockdown declined to 20\% in BT147p75NTR KD cells (Figures 3.2.C and D). These results showed that p75NTR expression was reduced dramatically upon transfection with a p75NTR shRNA targeting vector.
A

B

Mutational status of BT 48 and BT147

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<td>Primary GBM</td>
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<tr>
<td>p75NTR expression</td>
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C

Human Nucleolin/ p75NTR

Control (NO tumor)  BT147  BT48  BT69  BT191
Figure 3.1 BTIC express variable levels of $p75^{NTR}$

(A) Western blot analysis shows $p75^{NTR}$ expression in different patient-derived BTIC lines; BT147 and BT48 expressed high levels of $p75^{NTR}$. Actin was used as a loading control. (B) Table showing the mutational status of both BT48 and BT147. (Mut) indicates that mutations were detected in the tested gene, (Wt) indicates a wild-type gene and VIII indicates the detection of EGFRvIII in the tested BTIC line. (C) Immunofluorescence staining of paraffin-brain sections obtained from different BTIC xenografts shows that these BTICs express variable levels of $p75^{NTR}$ in vivo, where BT147 and BT48 express the highest $p75^{NTR}$ levels in the tested BTIC xenografts. Human nucleolin stain human nucleoli are red and $p75^{NTR}$ is green. All images were acquired by the IN Cell Analyzer 6000 imaging platform and using 40X objective. (D) RNA-seq analysis of the selected BTIC xenografts shows that variable $p75^{NTR}$ transcript levels in these BTIC xenografts with BT147 and BT48 show the highest levels of $p75^{NTR}$ transcripts among tested BTIC lines.
**Figure 3.2 Knockdown of p75<sup>NTR</sup> in genetically different BTICs**

(A) Western blot analysis shows p75<sup>NTR</sup> knockdown in BT147 and BT48. Actin was used as a loading control. (B) Immunocytochemical analysis of p75<sup>NTR</sup> shows low p75<sup>NTR</sup> levels (shown in red) in BT147 p75<sup>NTR</sup> KD and BT48 p75<sup>NTR</sup> KD compared to their controls. Cell nuclei were visualized with DAPI (blue). (C) Flow cytometric analysis of p75<sup>NTR</sup> expression in BT147p75<sup>NTR</sup> KD cells (green peak) and BT147 control cells (red peak) in the upper panel and BT48p75<sup>NTR</sup> KD cells (green peak) and BT48 control cells (red peak) in the lower panel. The p75<sup>NTR</sup> expression was set according to the isotype control (gray peak). (D) Quantification of three independent experiments of flow cytometric analysis of percentage of p75<sup>NTR</sup> expressing cells in p75<sup>NTR</sup> KD cells compared to controls in both BT147 (upper panel) and BT48 (lower panel). Values shown are the mean ± SEM. ** indicates $P < 0.01$ and *** indicates $P < 0.001$ (student t-test within a given sample).
3.2.2 Targeting p75<sup>NTR</sup> decreases sphere forming capacity and growth capacity of GBM patient-derived BTICs

Neurosphere formation is a defining characteristic of neural stem cells and cancer stem cells including brain tumor stem cells (Hemmati et al., 2003; Reynolds & Weiss, 1992; S. K. Singh et al., 2004) and is used to measure stem cell capacity for self-renewal. We determined neurosphere formation competence in cells with knockdown of p75<sup>NTR</sup>. Knockdown of p75<sup>NTR</sup> dramatically decreased neurosphere size of both BT147 and BT147 p75<sup>NTR</sup> KD compared to their control counterparts as seen in Figure 3.3.A. Using 10 µm as the smallest neurosphere size, we found knockdown of p75<sup>NTR</sup> significantly reduced the total number of neurospheres as well as the number of medium (20 - 50 µm) and large (50 ≥100 µm) neurospheres formed by both BT147 and BT48 p75<sup>NTR</sup> KD compared to their control counterparts (Figures 3.3.B and C). These results suggest that p75<sup>NTR</sup> is a key regulator of sphere-forming capacity of genetically different GBM patient-derived BTICs. To determine if the reduction in neurosphere formation capacity of p75<sup>NTR</sup> knockdown BTICs was due to decreased cell proliferation, we determined the proliferative capacity of both p75<sup>NTR</sup> knockdown cells and their control counterparts using AlamarBlue assay. In agreement with the results of the neurosphere formation assays, we found that p75<sup>NTR</sup> knockdown in both BT147 and BT48 cells dramatically decreased their proliferative capacity compared to their controls (Figure 3.4.A). To determine if the decrease in proliferation capacity of p75<sup>NTR</sup> knockdown resulted from a delay in cell cycle progression in these cells compared to their controls, we investigated the cell cycle progression by DNA content measurement in these cells. Flow cytometric analysis of the cell cycle with propidium iodide DNA staining showed a dramatic decreased in the percentage of cells in S-phase and a significant arrest in the G1-phase in both BT147 and BT48 p75<sup>NTR</sup> KD cells compared to their control counterparts (Figures 3.4.B and
C). These results suggest that p75<sup>NTR</sup> regulates proliferation of BTICs, and p75<sup>NTR</sup> knockdown results in a delay in the cell cycle progression.
A

Control  KD

BT147

BT48

B

Number of neurosphere >10µM

Control  KD

BT147

***

BT48

***

C

Number of neurospheres

Control  KD

BT147

***

BT48

***
Figure 3.3 Knockdown of $p75^{NTR}$ reduced sphere-forming capacity of genetically different GBM patient-derived BTICs

(A) Representative images of neurospheres formed by BT147 control or BT147$p75^{NTR}$ KD cells in the upper panel and BT48 control and BT48$p75^{NTR}$ KD cells. Knockdown of $p75^{NTR}$ reduced the size of spheres in both BT147 and BT48. Images were acquired using an IN Cell Analyzer 6000 with 10X objective. (B) Quantification of three independent experiments of the total number of neurospheres $>10\mu m$ formed by BT147 control and BT147$p75^{NTR}$ KD cells (left panel) or BT48 control and BT48$p75^{NTR}$ KD cells (right panel). Knockdown of $p75^{NTR}$ resulted in fewer neurospheres $>10\mu m$ compared to control cells in both BT147 and BT48. (C) Quantification of three independent experiments that measured the size of neurospheres ranging from $10-100\mu m$ formed by BT147 control and BT147$p75^{NTR}$ KD cells (left panel) or BT48 control and BT48$p75^{NTR}$ KD cells (right panel). Knockdown of $p75^{NTR}$ reduced the overall size of neurospheres in both BT147 and BT48. Values show the mean ± SEM from 3 independent experiments; NS indicates no statistical significance; *** indicates $P < 0.0001$; ** indicates $P < 0.001$, Analysis was performed with 2-way ANOVA with Bonferroni’s multiple comparisons test.
### A

**BT147**

- **Control**
  - G1: 79.80%
  - G2: 6.69%
  - S: 13.50%

- **KD**
  - G1: 66.02%
  - G2: 5.46%
  - S: 27.96%

**BT48**

- **Control**
  - G1: 69.13%
  - G2: 3.15%
  - S: 27.72%

- **KD**
  - G1: 84.19%
  - G2: 3.49%
  - S: 12.32%

### B

**BT147**

- **Control**
  - G1: 66.02%
  - G2: 5.46%
  - S: 27.96%

- **KD**
  - G1: 79.80%
  - G2: 6.69%
  - S: 13.50%

**BT48**

- **Control**
  - G1: 69.13%
  - G2: 3.15%
  - S: 27.72%

- **KD**
  - G1: 84.19%
  - G2: 3.49%
  - S: 12.32%

### C

**BT147**

- **Control**
  - G1: 66.02%
  - G2: 5.46%
  - S: 27.96%

- **KD**
  - G1: 79.80%
  - G2: 6.69%
  - S: 13.50%

**BT48**

- **Control**
  - G1: 69.13%
  - G2: 3.15%
  - S: 27.72%

- **KD**
  - G1: 84.19%
  - G2: 3.49%
  - S: 12.32%
Figure 3.4 Knockdown of p75NTR reduced proliferation and induced G1 cell cycle arrest in genetically different GBM patient-derived BTICs

(A) Cell proliferation of BT147 control and BT147p75NTR KD (left panel) and BT48 control and BT48p75NTR KD (right panel) was assessed by AlamarBlue assay. BTICs were plated in a 96 well-plate (5000 cells/well) for 5 days. On day 5, AlamarBlue was added to the cells and absorbance measured 6 hours later. Values shown are the mean ± SEM from 3 independent experiments. *** indicates $P < 0.001$ (student t-test within a given sample). (B) Cell cycle analysis of BT147 control and BT147p75NTR KD (upper panel) and BT48 control and BT48p75NTR KD (lower panel). Knockdown of p75NTR in both BT147 and BT48 induced accumulation of p75NTR KD cells in the G0/G1 cell cycle phase. Data shown are from one of three independent experiments. (C) quantification of cell cycle analysis of BT147 control and BT147p75NTR KD (upper panel) or BT48 control and BT48p75NTR KD (lower panel). Values shown are the mean ± SEM from three independent experiments. NS indicates no statistical significance; *** indicates $P < 0.0001$, ** indicates $P < 0.001$. Analysis was performed with 2-way ANOVA with Bonferroni’s multiple comparisons test.
3.2.3 Targeting p75\(^{\text{NTR}}\) in GBM patient-derived BTICs suppresses glioma growth \textit{in vivo}

The most important property of BTICs is their potent ability to propagate tumors \textit{in vivo}. Because the p75\(^{\text{NTR}}\) knockdown dramatically decreased sphere-forming capacity and the proliferation capacity of BTIC \textit{in vitro}, we sought to determine if targeting p75\(^{\text{NTR}}\) expression could attenuate the tumorigenic potential of these BTICs. To accomplish this goal, 100,000 cells of p75\(^{\text{NTR}}\) knockdown cell BT147 and BT48 p75\(^{\text{NTR}}\) KD cells and their control counterparts were implanted into the brains of SCID mice to form tumors. Based on a previous characterization of these cells \textit{in vivo}, mice were sacrificed at 7 weeks for BT147 control and BT147p75\(^{\text{NTR}}\) KD and at 4 months for BT48 control and BT48p75\(^{\text{NTR}}\) KD. The mice were sacrificed at the designated time, and paraffin-embedded brain sections were prepared for analysis. Since implanted cells were transfected previously with GFP-construct, paraffin-embedded brain sections from both BTIC p75\(^{\text{NTR}}\) knockdown xenografts and their control counterparts were used to assess the tumor growth under fluorescent microscopy. These sections were imaged using an IN Cell 6000 Analyzer and stitched images of the whole brain section showed that only a few GFP (green) positive cells were detected in the brain tumors formed by the BTIC p75\(^{\text{NTR}}\) knockdown (BT147 and BT48 p75\(^{\text{NTR}}\) KD); these cells were located mainly around the injection sites. In contrast, control BTICs formed highly infiltrative tumors, and GFP (green) cells were detected invading through the brain parenchyma and along the corpus callosum (Figure 3.5.A). To determine if the dramatic tumor growth delay of BT147 and BT48 p75\(^{\text{NTR}}\) was the result of the p75\(^{\text{NTR}}\) knockdown, whole-brain sections from mice bearing orthotopic patient-derived xenografts (BT147 and BT48 p75\(^{\text{NTR}}\) KD) and their control counterparts were immunofluorescently stained for p75\(^{\text{NTR}}\) (green) and human nucleolin (red) to detect the human tumor cells (Figure 3.5.B). The immunofluorescent staining analysis revealed a dramatic reduction of p75\(^{\text{NTR}}\) expression on tumor cells of BT147 and BT48...
p75\textsuperscript{NTR} KD compared to their controls BT147 and BT48 respectively (Figure 3.5.B). To determine if the delay of tumor growth of p75\textsuperscript{NTR} knockdown BTICs resulted from low proliferative capacity of these cells, as we observed \textit{in vitro} (Figure 3.4.A), we measured the percentage of proliferative cells using a Bromodeoxyuridine (BrdU) incorporating approach. Mice bearing brain tumors formed by p75\textsuperscript{NTR} knockdown BTICs or by their control counterparts were intraperitoneally injected with BrdU to label proliferating cells 24 hours before sacrificing the mice. Immunofluorescent staining of BrdU positive cells in paraffin-embedded brain sections obtained from these mice showed a reduction in the number of proliferative tumor cells (BrdU, green) in BT147 and BT48 p75\textsuperscript{NTR} KD xenografts compared to proliferative cells detected in xenografts of their controls as shown in Figure 3.6.A and quantified in Figure 3.6.B. These results suggest that p75\textsuperscript{NTR} is a key regulator of proliferation of GBM patient-derived BTICs. To determine if the reduction in the percentage of proliferative cells and the delay of tumor growth in p75\textsuperscript{NTR} knockdown BTICs was due to decreased cell survival, we determined the percentage of apoptotic cells in the whole brain sections of mice bearing brain tumors formed by p75\textsuperscript{NTR} knockdown BTICs or by their control counterparts using TUNEL staining. The analysis of these sections showed a significant increase of the percentage of apoptotic tumor cells in BT147 and BT48 p75\textsuperscript{NTR} KD xenografts compared to their control counterparts as shown in Figure 3.7.A and quantified in Figure 3.7.B. Kaplan Meir studies were performed to determine whether the delayed tumor growth that resulted from p75\textsuperscript{NTR} knockdown in BTICs had an impact on the overall survival. Mice bearing brain tumors formed by p75\textsuperscript{NTR} knockdown BTICs or by their control counterparts were recruited for survival studies and monitored until they developed neurological sickness signs according to our animal ethics protocol (number AC14.0107). Interestingly, p75\textsuperscript{NTR} knockdown in BT147 and BT48 extended the survival time of mice bearing brain tumors formed
by these cells compared to mice bearing brain tumors formed by BT147 and BT48 controls (Figure 3.8.A). Although p75\textsuperscript{NTR} knockdown BTICs were able eventually to form large tumors, these tumors were not formed by cells that escaped p75\textsuperscript{NTR} knockdown because p75\textsuperscript{NTR} expression on these cells was not detected (Figure 3.8.B). Furthermore, tumors formed by p75\textsuperscript{NTR} knockdown BTICs showed lower tumor cell density compared to tumors formed by control BTICs (Figure 3.8.C). Taken together, these data demonstrate that p75\textsuperscript{NTR} is required for maintaining the tumorigenic capacity of BTICs \textit{in vivo}. 
Figure 3.5 Knockdown of p75NTR in patient-derived BTIC reduced their tumorigenicity *in vivo*

(A) Whole-brain sections from animals implanted with BT147 control and BT147p75NTR KD (upper panels) or BT48 control and BT48p75NTR KD cells (lower panels). Tumor cells expressing a GFP construct were imaged using an IN Cell Analyzer 6000. Both BT147 and BT48 control cells formed highly infiltrative tumor cells (GFP; green) seen invading through the brain parenchyma and along the corpus callosum. Only a few tumor cells were detected in the brain section of animals implanted with BT147p75NTR KD or BT48p75NTR KD as indicated by the white triangles. (B) Whole-brain sections from animals bearing orthotopic patient-derived xenografts. BT147 control and BT147p75NTR KD cells (upper panels) or BT48 control and BT48p75NTR KD cells (lower panels) were stained with anti-p75NTR (green) and a human-specific nucleolin antibody (red) to detect the human tumor cells. Expression of p75NTR was dramatically reduced in both BT147p75NTR KD and BT48p75NTR KD cells compared to BT147 and BT48 control cells.
A

**Brdu**

**Human Nucleolin**

**overlap**

Control

BT147

KD

Control

BT48

KD

B

**BT147**

**BT48**

![Graphs for BT147 and BT48 showing percentage of proliferative cells (BrdU).](image)
Figure 3.6 Knockdown of p75NTR in patient-derived BTICs inhibited their proliferation in vivo

(A) Immunofluorescence images of Bromodeoxyuridine (BrdU) positive cells (green) in whole brain sections from animals implanted with BT147 control and BT147p75NTR KD cells (upper panels) or BT48 control and BT48p75NTR KD cells (lower panels). BrdU was injected into the animals 24 hours before sacrificing them. Brain sections were stained with anti-BrdU (green) and a human-specific nucleolin antibody (red) to detect the human tumor cells. Images were acquired by an IN Cell Analyzer 6000 with 20X objective. Knockdown of p75NTR reduced the percentage of proliferative cells in both BT147 and BT48. (B) Quantification of BrdU positive tumor cells in whole brain sections from animals implanted with BT147 control and BT147p75NTR KD cells (left panel) or BT48 control and BT48p75NTR KD cells (right panel). Values shown are the mean ± SEM from three independent experiments. *** indicates $P < 0.0001$; ** indicates $P < 0.001$. Analysis was performed with Student t-test.
Figure 3.7 Knockdown of p75\textsuperscript{NTR} in patient-derived BTICs induced apoptotic cell death \textit{in vivo}

(A) Immunofluorescence images of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) positive cells (green) in whole brain sections from mice implanted with BT147 control and BT147p75\textsuperscript{NTR} KD cells (upper panels) or BT48 control and BT48p75\textsuperscript{NTR} KD cells (lower panels). Whole brain sections were co-stained for the presence of apoptosis by TUNEL (green) and a human-specific nucleolin antibody (red) to detect the human tumor cells. Images were acquired by an IN Cell Analyzer 6000 with 20X objective. Knockdown of p75\textsuperscript{NTR} increased the percentage of apoptotic cells in both BT147 and BT48. (B) Quantification of TUNEL positive tumor cells in whole brain sections from mice implanted with BT147 control and BT147p75\textsuperscript{NTR} KD cells (left panel) or BT48 control and BT48p75\textsuperscript{NTR} KD cells (right panel). Values shown are the mean ± SEM from three independent experiments. * indicates $P < 0.01$. Analysis was performed with Student t-test.
A

![Graphs showing percent survival for BT147 and BT48 with Control and KD groups.](image)

- **BT147**
  - Control: Green line
  - KD: Red line
  - Weeks: 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24
  - Percent survival: 0, 20, 40, 60, 80, 100
  - **p = 0.0084**

- **BT48**
  - Control: Green line
  - KD: Red line
  - Months: 0, 5, 10, 15, 20
  - Percent survival: 0, 50, 100
  - **p = 0.0058**

B

**BT147**

- Control 7 weeks
- KD 7 weeks
- KD End Stage

**BT48**

- Control 4 months
- KD 4 months
- KD End stage
Figure 3.8 Knockdown of p75\textsuperscript{NTR} in patient-derived BTICs extended survival of mice bearing BTIC-derived xenografts

(A) Kaplan-Meier survival curves of mice implanted with BT147 control and BT147p75\textsuperscript{NTR} KD cells (left panel) or BT48 control and BT48p75\textsuperscript{NTR} KD cells (right panel). Mice bearing brain tumors generated by BT147p75\textsuperscript{NTR} KD or BT48p75\textsuperscript{NTR} KD showed a significant survival extension relative to mice implanted with BT147 and BT48 control cells. ** indicates $P < 0.001$. (n= 4 mice in each group). (B) Immunofluorescent analysis of p75\textsuperscript{NTR} (green) in brain sections of mice bearing brain tumors formed by BT147 control or BT147p75\textsuperscript{NTR} KD cells (upper panels) or BT48 control or BT48p75\textsuperscript{NTR} KD cells (lower panels). Mice were sacrificed at the same time points (7 weeks for animals bearing tumors formed by BT147 control and BT147p75\textsuperscript{NTR} KD cells as early stage and 22 weeks as end stage of animals bearing tumors formed by BT147p75\textsuperscript{NTR} KD cells) or 4 months for animals bearing tumors formed by BT48 control and BT48p75\textsuperscript{NTR} KD cells as early stage or at 17 months as end stage of animals bearing tumors formed by BT48p75\textsuperscript{NTR} KD cells. Whole paraffin-embedded brain sections from these animals were stained with anti-p75\textsuperscript{NTR} (green) and a human-specific nucleolin antibody (red) to detect the human tumor cells. Marked reduction of p75\textsuperscript{NTR} expression was observed in tumor cells of both early and late stages tumors formed by BT147p75\textsuperscript{NTR} cells compared to BT147 control cells (upper panels) or BT48p75\textsuperscript{NTR} KD cells compared to BT48 control cells (lower panels). Images were acquired by an IN Cell Analyzer 6000 with 40X objective. (C) Immunofluorescent analysis of human nucleolin (red) to detect the human tumor cells in brain sections of mice bearing tumors formed by BT147 control or BT147p75\textsuperscript{NTR} KD cells (upper panels) or BT48 control or BT48p75\textsuperscript{NTR} KD cells (lower panels). All animals were sacrificed when they developed sickness signs (end stages). Mice bearing tumors formed by BT147 control cells were sacrificed at 10 weeks and those bearing tumors formed by BT147p75\textsuperscript{NTR} KD cells were sacrificed at 22 weeks. Mice bearing tumors formed by BT48 control cells were sacrificed at 8 months and those bearing tumors formed by BT48p75\textsuperscript{NTR} KD control cells were sacrificed at 17 months. Knockdown of p75\textsuperscript{NTR} reduced tumor cell density compared to control BTICs in both BT147 and BT48. Images were acquired by an IN Cell Analyzer 6000 with 4x objective and stitched using IN Cell Analyzer developer software.
3.2.4 Targeting p75$^{NTR}$ in GBM patient-derived BTICs disrupts their symmetric and asymmetric cell divisions

Stem and progenitor cells are identified by two main characteristics: self-renewal ability and production of differentiated progeny. A controlled balance between these processes is accomplished through regulated asymmetric divisions and is crucial to produce cellular diversity during development and to maintain adult tissue homeostasis (Figure 3.9.A). Disruption of this balance may cause premature depletion of the pool of stem/progenitor cells, or lead to abnormal growth (Gomez-Lopez, Lerner, & Petritsch, 2014). Symmetric and asymmetric cell divisions play a role in maintaining glioma stem cells pool through distribution of CD133 and Numb (Lathia et al., 2011). Because we observed that p75$^{NTR}$ knockdown inhibited the sphere-forming capacity and proliferation ability of BTICs, we sought to determine if it is also changes the balance of the BTICs pool through disrupting symmetric and asymmetric cell divisions to produce more differentiated progenies. To achieve this goal, single cells of p75$^{NTR}$ knockdown BTICs or their controls were plated at a low density (1000 cell) on glass coverslips coated with the extracellular matrix (ECM) protein, laminin, as a substrate, previously shown to facilitate long-term adherent culture (Pollard et al., 2009). After 24–30 hours (time of cell division was observed under the microscope), cells were fixed and symmetry and asymmetry of cell division of the BTICs were analyzed using Numb, which has been implicated in regulating symmetry and asymmetry cell division in neural stem cells (Shen, Zhong, Jan, & Temple, 2002). During asymmetry cell division, Numb segregates into the differentiating daughter cell, where it inhibits self-renewal (Lee et al., 2006; Rhyu, Jan, & Jan, 1994). Cell symmetry and cell asymmetry cell division of p75$^{NTR}$ knockdown BTICs that were analyzed by Numb expression is shown in Figure 3.9.B. Quantification of symmetry and asymmetry cell divisions showed that p75$^{NTR}$ knockdown in BT147 and BT48 cells significantly increased symmetric cell division, where both daughter cells
expressed Numb compared to the BT147 and BT48 control cells (Figure 3.9.C). These results suggest that p75NTR knockdown decreased the stem cell pool by increasing differentiating progenies.
A

- Stem cell
  - Symmetric cell division
  - Asymmetric cell division
  - Differentiating cells
    - Numb +ve
    - Numb -ve

B

- DAPI
- Numb
- merge

C

- BT147 Control
- BT147 KD
- BT48 Control
- BT48 KD

```plaintext
ns
***
```
Figure 3.9 Knockdown of p75NTR in patient-derived BTICs disrupted their symmetric and asymmetric cell divisions

(A) A schematic figure showing the symmetry and asymmetry stem cell divisions. Stem cells can undergo symmetry cell divisions, where a stem cell can give rise to two daughter stem cells that have self-renewal ability, or asymmetry cell division, where a stem cell can give rise to a stem cell and a differentiating cell that expresses the determinant cell fate protein (Numb), which inhibits self-renewal ability. A stem cell can also divide symmetrically to give rise to two daughter-differentiating cells that can develop to mature differentiated cells. (B) Immunofluorescent analysis of Numb expression (red) in a dividing stem cell (BT147) to show asymmetry cell division where a stem cell give rise to a stem cell (Numb –ve) and a differentiating cell (Numb +ve). In symmetric cell divisions, a stem cell can give rise to two daughter stem cells (both Numb –ve) or a stem cell give rise to two differentiating cells (both Numb +ve). Cell nuclei were visualized with DAPI. Images were acquired by an IN Cell Analyzer 6000 using 40X objective. (C) Quantification of symmetry and asymmetry cell divisions of BT147 control and BT147p75NTR KD cells (left panel) and BT48 control and BT48p75NTR KD cells (right panel). Knockdown of p75NTR in both BT147 and BT48 increased percentage of symmetric cell division to give rise to more differentiating cells (Numb +ve) and reduced percentage of symmetric cell division to give rise to more stem cells (Numb -ve). Values shown are the mean ± SEM from three independent experiments. NS indicates no statistical significance; *** indicates P < 0.0001. Analysis was performed with 2-way ANOVA with Bonferroni’s multiple comparisons test.
3.2.5 p75<sup>NTR</sup> knockdown induces expression of differentiation-associated proteins

Another defining characteristic of neural stem cells (NSCs) is their multipotency, the ability to differentiate into neurons, astrocytes, and oligodendrocytes (Reynolds & Weiss, 1992). Because p75<sup>NTR</sup> knockdown resulted in changing the stem cell pool in BTICs and producing more differentiating progenies, we sought to determine whether p75<sup>NTR</sup> knockdown is able to induce BTICs differentiation toward astrocytic, neural or oligodendroglial lineages. To achieve this goal, we tested the presence of differentiating cells in tumors generated by p75<sup>NTR</sup> knockdown BTICs and their control counterparts. Immunofluorescence staining of paraffin-brain sections of these tumors revealed striking morphological changes of the astrocytic marker, glial fibrillary acidic protein (GFAP)-expressing cells with long and marked processes in tumors generated by p75<sup>NTR</sup> knockdown BTICs and their control counterparts (Figure 3.10.A). To test whether these GFAP-expressing cells are differentiating tumor cells or they are activated astrocytes in the tumor microenvironment, we performed multiplex immunostaining for GFAP, human nucleolin and counterstaining with DAPI. GFAP-expressing cells with both human nucleolin and DAPI positive staining indicates that these cells are differentiating tumor cells while GFAP-expressing cells without human nucleolin staining indicates they are activated astrocytes in the tumor microenvironment. Multiplex immunostaining analysis showed the presence of both differentiating tumor cells and activated astrocytes in both BT147 and BT48 p75<sup>NTR</sup> knockdown tumors compared to their controls (Figure 3.10.B). Multiplex immunostaining of ionized calcium-binding adapter molecule 1 (Iba1), a microglia/macrophage marker, human nucleolin and DAPI were used as a negative control (Figure 3.10.C). The stitched images of the whole brain sections of mice bearing tumors generated by p75<sup>NTR</sup> knockdown BTICs or their control counterparts showed a dramatic increase in the percentage of GFAP-expressing cells in both BT147 and BT48.
p75NTR knockdown tumors compared to their controls (Figure 3.10.D). To test whether p75NTR knockdown can induce morphological changes of differentiating BTICs into astrocytic lineage in vitro, both BT147 and BT48 p75NTR knockdown cells and their control counterparts were incubated in neural stem cell medium containing 2% of FBS for 7 days to allow cells to differentiate towards any of the neural lineages. After 7 days, cells were washed, fixed and processed for immunocytochemical studies. As we observed in vivo, immunocytochemical analysis showed that GFAP-expressing cells of BT147 and BT48 p75NTR knockdown have different morphologies accompanied with the presence of long processes of these cells compared to GFAP-expressing cells in BT147 and BT48 controls (Figure 3.10.E). Western blot analysis showed a dramatic increase of GFAP protein levels in p75NTR knockdown BTICs compared to their control counterparts (Figure 3.10.F). Collectively, these results suggest that p75NTR knockdown in BTICs induced their differentiation towards astrocytic lineage.
A

Control

BT147

KD

Control

BT48

KD

Human Nucleolin

GFAP

Merge

B

Control

BT147

KD

Control

BT48

KD

Activated Astrocytes

Differentiating tumor cells

GFAP

Human nucleolin

DAPI

merge

Activated Astrocytes

Differentiating tumor cells
Figure 3.10 Knockdown of p75NTR induced BTICs differentiation towards astrocytic lineage

(A) Immunofluorescent analysis of GFAP (green) in brain sections of mice bearing brain tumors formed by BT147 control or BT147p75NTR KD cells (upper panels) or BT48 control or BT48p75NTR KD cells (lower panels). Knockdown of p75NTR induced morphological changes (star-shaped) of GFAP-expressing cells in tumors generated by both BT147p75NTR KD and BT48p75NTR KD compared to their controls. Human-specific nucleolin antibody (red) was used to detect the human tumor cells. Images were acquired by an IN Cell Analyzer 6000 using 40X objective. (B) Multiplex immunofluorescent analysis of GFAP (green), human nucleolin (red) and DAPI (blue) showed that the GFAP-expressing cells in tumors generated by BT147p75NTR KD (upper panels) and BT48p75NTR KD cells (lower panels) have either nuclei that are positive for DAPI only, indicating they are activated astrocytes (right upper panel) or positive for both for human nucleolin and DAPI, indicating they are differentiating tumor cells (right lower panel). (C) Immunofluorescent staining for Ib1 was used as a negative control. Images were acquired by an IN Cell Analyzer 6000 with 40X objective. (D) Immunofluorescent analysis of GFAP (green) in whole brain sections of mice bearing brain tumors formed by BT147 control or BT147p75NTR KD cells (upper panels) or BT48 control or BT48p75NTR KD cells (lower panels). Human-specific nucleolin antibody (red) was used to detect the human tumor cells. Images were acquired by an IN Cell Analyzer 6000 with 4X objective and stitched using IN Cell Analyzer developer software. (E) Immunofluorescent analysis of GFAP-expressing cells (red) in BT147 control and BT147p75NTR KD cells (upper panels) and BT48 control and BT48p75NTR KD cells (lower panel) grown in the presence of 2% FBS for 7 days. Knockdown of p75NTR induced morphological changes (star-shaped) of GFAP-expressing cells in both BT147 and BT48 compared to their controls. Images were acquired by an IN Cell Analyzer 6000 with 40X objective. (F) Western blot analysis of BT147 control and BT147p75NTR KD (left panel) and BT48 control and BT48p75NTR KD (right panel) grown in 2% FBS for 7 days shows increased GFAP expression levels in BT147p75NTR KD and BT48p75NTR KD cells compared to BT147 control and BT48 control cells, respectively. Actin was used as a loading control.
3.2.6. \( p75^{\text{NTR}} \) knockdown suppresses the expression of cancer stem cell markers

GBM tumors with heterogeneous cellular composition include cellular subpopulations that express neural stem and progenitor cell markers including Nestin SOX2, Olig2, CD133, CD15 and CD44 (Lathia et al., 2010; Ligon et al., 2007; S. K. Singh et al., 2004; Son et al., 2009). The increase in the expression of the astrocytic marker, GFAP, led us to further analyze the effects of \( p75^{\text{NTR}} \) knockdown on the expression of neural stem and progenitor cell markers that have been previously shown to be expressed by BTICs. Immunohistochemical and immunofluorescence analysis of brain sections of \( p75^{\text{NTR}} \) knockdown tumors and their control counterparts showed a dramatic reduction in the expression of Olig2 and Nestin in \( p75^{\text{NTR}} \) knockdown tumors compared to their controls (Figure 3.11.A and B). Furthermore, multiplex immunostaining analysis of SOX2 and GFAP in tumors generated by \( p75^{\text{NTR}} \) knockdown BTICs or their control counterparts showed that \( p75^{\text{NTR}} \) knockdown cells express high levels of GFAP and low levels of SOX2, but, on the contrary, control BTICs express low levels of GFAP and high levels of SOX2 (Figure 3.11.C). These results were confirmed by immunoblot analysis of \( p75^{\text{NTR}} \) knockdown BTICs and their control grown in vitro (Figure 3.11.D). Taken together, these observations suggest that \( p75^{\text{NTR}} \) correlates with the stemness status of BTICs, and \( p75^{\text{NTR}} \) depletion in these cells induces their differentiation towards an astrocytic lineage.
Figure 3.11 Knockdown of p75<sup>NTR</sup> in patient derived BTICs suppresses stem cell markers

(A) Immunofluorescent analysis of Olig2 (green) in brain sections of mice bearing brain tumors formed by BT147 control or BT147p75<sup>NTR</sup> KD cells (upper panels) or BT48 control or BT48p75<sup>NTR</sup> KD cells (lower panels). Human-specific nucleolin antibody (red) was used to detect the human tumor cells. Knockdown of p75<sup>NTR</sup> reduced the percentage of cells expressing Olig2 in both BT147 and BT48. Images were acquired by an IN Cell Analyzer 6000 using 40X objective.

(B) Immunohistochemical analysis of Nestin (brown) expression in BT147 control and BT147p75<sup>NTR</sup> KD cells (upper panels) and BT48 control and BT48p75<sup>NTR</sup> KD cells (lower panels). Knockdown of p75<sup>NTR</sup> reduced the Nestin expression in tumors formed by both BT147p75<sup>NTR</sup> KD and BT48p75<sup>NTR</sup> KD cells compared to that of tumors formed by BT147 control and BT48 control cells. Scale bar is 40 µm.

(C) Multiplex immunofluorescent analysis of GFAP (green), SOX2 (white) and human nucleolin (red) in brain sections of mice bearing brain tumors formed by BT147 control or BT147p75<sup>NTR</sup> KD cells (upper panels) or BT48 control or BT48p75<sup>NTR</sup> KD cells (lower panels). Knockdown of p75<sup>NTR</sup> reduced SOX2 expression and increased GFAP expression in tumors formed by both BT147p75<sup>NTR</sup> KD and BT48p75<sup>NTR</sup> KD cells compared to expression in tumors formed by BT147 control and BT48 control cells. Images were acquired by an IN Cell Analyzer 6000 using 40X objective.

(D) Western blot analysis shows a reduction in expression levels of p75<sup>NTR</sup>, SOX2 and Olig2 in BT147 p75<sup>NTR</sup> KD cells compared to expression levels in BT147 control cells (left panel) and in BT48p75<sup>NTR</sup> KD cells compared to that in BT48 control cells. Actin was used as a loading control.
3.2.6 Defining *in vivo* molecular processes and transcriptional changes mediated by p75NTR knockdown

To better understand the molecular processes involved in inhibiting proliferation, reducing the expression of stem cell associated markers, inducing cell death and attenuating tumorigenicity of p75NTR knockdown BTICs compared to their controls in the tumor microenvironment, RNA was isolated from brain tumors generated by BT147 p75NTR knockdown (3 mice) and their controls BT147 (3 mice) and processed for global gene expression analysis (*Figure 3.12.A*). Gene level differential expression analysis was performed using Affymetrix PrimeView human gene expression array. We used fold change < -2 and >2 as a cuttoff and out of 49,372 identified genes, found 5,131 genes that were differentially expressed between tumor cells isolated from animals bearing tumors formed by BT147p75NTR knockdown or BT147 control cells. Of those 5,131 genes, we found 3,621 genes that were downregulated and 1,510 genes that were upregulated in BT147p75NTR knockdown cells compared to BT147 control cells. By using Ingenuity Pathway Analysis (IPA®) software (version 01-12; Qiagen) to identify changes in the pathways and biological processes that resulted from p75NTR knockdown in BT147 we observed downregulation of genes associated with different pathways involved in cell survival, migration and invasion of tumor cells, cell cycle progression and proliferation of stem and tumor cells, and upregulation of genes associated with increased cell death, apoptosis of tumor cells, and growth inhibition (*Figure 3.12.B*). These results confirm our findings regarding the role of p75NTR in the biology of BTICs both *in vitro* and *in vivo*. Close examination of the affected pathways in p75NTR knockdown cells showed a universal downregulation of genes involved in cell cycle progression (*Figure 3.12.C and D*). This downregulation provides a possible reason of the delay of tumor growth observed in mice bearing tumors formed by p75NTR knockdown cells. In addition to the above described pathways we also found changes in several other interesting pathways involved in regulation of
metabolism, inflammation, apoptosis, angiogenesis and extracellular matrix modulation among others. The correlation between p75NTR and changes in these pathways would need to be explored in future studies.
Intracranial injection of BT147 Control or BT147p75p75 KD

Sacrificed when develop sickness signs

Freeze at -80°C

Step: 1

BT147 Control
BT147p75p75 KD

Step: 2

mRNA

RNA isolation

Reverse transcriptase labelling

cDNA

Hybridize to Microarray

scanning

Data Analysis

Analysis Comparison 1

Activation z-score

Pathways and biological processes

Cell survival
Invasion of cells
Growth Failure
Cell transformation
Apoptosis
Migration of tumor cells
S phase
DNA replication
Invasion of tumor cells
Repair of DNA
Angiogenesis
Cell cycle progression
Progression of tumor
Proliferation of stem cells
Proliferation of tumor cells
Cell death of tumor cells
Colony formation of cells
Senescence of cells
Apoptosis of tumor cells
C

Cell Cycle related pathways

- **Cell Cycle**: 33 downregulated, 13 upregulated
- **G1 to S cell cycle control**: 21 downregulated, 2 upregulated
- **Mitotic G2-G2/M phases**: 14 downregulated, 19 upregulated
- **DNA Replication**: 1 downregulated, 1 upregulated

D

Cell Cycle related pathways

- **Cell Cycle**: 33 downregulated, 13 upregulated
- **G1 to S cell cycle control**: 21 downregulated, 2 upregulated
- **Mitotic G2-G2/M phases**: 14 downregulated, 19 upregulated
- **DNA Replication**: 1 downregulated, 1 upregulated

Green = down-regulated genes in p75<sup>sh</sup> KD cells
Red = up-regulated genes in p75<sup>sh</sup> KD cells
Figure 3.12 Gene expression analysis of tumor cells isolated from brains of animals bearing tumors formed by BT147 control and BT147p75^{NTR} KD cells

(A) A schematic figure of microarray analysis steps of tumors formed by BT147 control and BT147p75^{NTR} KD cells. BT147 control or BT147p75^{NTR} KD cells were implanted into the brains of SCID mice (100,000 cell/mouse). When the mice showed sickness signs, they were sacrificed and brains were isolated and cut into two halves (one half that contains the tumor mass and the other half that may contain invasive tumor cells) and incubated at $-80^\circ$C. RNA was isolated from the halfbrain that contained the tumor and used to prepare cDNA. cDNAs were hybridized to microarray chips specific for human gene detection. Microarray chips were scanned and the data analyzed using Ingenuity® Pathway Analysis (IPA®) software. (B) A heatmap of pathways and biological processes that upregulated or downregulated in BT147 control and BT147p75^{NTR} KD cells based on gene expression analysis involved in these pathways. Orange color indicates high gene expression activity and blue color indicates low activity. A fold-change cut-off of 2 and P-value threshold of 0.05 were used to determine differential gene expression. (C) IPA® of cell cycle related pathways in BT147 control and BT147p75^{NTR} KD cells. Blue color indicates downregulated genes in BT147p75^{NTR} KD cells and orange color indicates upregulated genes in BT147p75^{NTR} KD cells. The numerical value indicates the number of genes that downregulated or upregulated in BT147p75^{NTR} KD cells compared to BT147 control cells. (D) A schematic figure shows cell cycle phases with downregulated or upregulated genes in BT147p75^{NTR} KD cells compared to BT147 control cells. Green color indicates downregulated genes and red color indicates upregulated genes.
3.3.1 p75NTR overexpression increased sphere-forming capacity and stem cell associated markers of GBM patient-derived BTICs

To further investigate the role of p75NTR in the biology of BTICs, p75NTR gain of function studies were performed. Genetically different GBM patient-derived BT73 and BT206 lines that lacked endogenous p75NTR expression were stably transfected with the full-length cDNA of human p75NTR or with empty PiggyBac vectors as controls. Expression levels of p75NTR in these cells were confirmed by western blot (Figure 3.13.A) and immunofluorescence staining (Figure 3.13.B). To determine if the p75NTR ectopic expression increased stem cell characteristics in BTICs, these cells were assessed for their sphere-forming capacity. In contrast to the suppression of sphere-forming capacity of p75NTR-depleted BTICs, p75NTR ectopic expression in BTICs increased the size of their neurospheres as shown in Figure 3.13.C and quantified in Figure 3.13.D, and increased the number of these neurospheres (Figure 3.13.E). These results suggest that p75NTR is a key regulator of BTIC sphere-forming capacity. We further investigated whether ectopic expression of p75NTR in BTICs is associated with increases in stem cell markers CD133 and CD15. Flow cytometry analysis of CD15 and CD133 in BT73p75NTR and BT206p75NTR and their control counterparts showed a dramatic increase of these markers in p75NTR-expressing BTICs (Figures 3.14.A and B), suggesting an important role of p75NTR in maintaining stem cell characteristics of these cells.
Figure 3.13 p75\textsuperscript{NTR} overexpression in patient-derived BTICs enhanced their sphere-forming capacity

(A) Western blot analysis of p75\textsuperscript{NTR} shows expression of p75\textsuperscript{NTR} in BT73 and BT206 cells stably transfected with PiggyBac vector encoding human p75\textsuperscript{NTR} (BT73p75\textsuperscript{NTR} and BT206p75\textsuperscript{NTR}) or empty vector as control (BT73 and BT206 controls). Actin was used as a loading control. (B) Immunofluorescent analysis of p75\textsuperscript{NTR} confirmed p75\textsuperscript{NTR} expression (red) in BT73p75\textsuperscript{NTR} compared to BT73 control cells (upper panels) and in BT206p75\textsuperscript{NTR} compared to BT206 control cells (lower panels). Cell nuclei were visualized with DAPI. Images were acquired by an IN Cell Analyzer 6000 using 40X objective. (C) Representative images of neurospheres formed by BT73 control and BT73p75\textsuperscript{NTR} cell (upper panels) and BT206 control and BT206p75\textsuperscript{NTR} cells (lower panels). Overexpression of p75\textsuperscript{NTR} in both BT73 and BT206 increased the size of neurospheres. Images were acquired by an IN Cell Analyzer 6000 using 10X objective. (D) Quantification of the size of neurospheres ranged from 10–100\(\mu\)m formed by BT73 control and BT73p75\textsuperscript{NTR} cells (left panel) or BT206 control and BT206p75\textsuperscript{NTR} cells (right panel). Overexpression of p75\textsuperscript{NTR} in both BT73 and BT206 increased the percentage of neurospheres >100\(\mu\)m. Values shown are the mean \(\pm\) SEM from three independent experiments. NS indicates no statistical significance; *** indicates \(P< 0.0001\). Analysis was performed with 2-way ANOVA with Bonferroni’s multiple comparisons test. (E) Quantification of the number of neurospheres >10\(\mu\)m formed by BT73 control and BT73 p75\textsuperscript{NTR} cells (left panel) or BT206 control and BT206p75\textsuperscript{NTR} cells (right panel). Overexpression of p75\textsuperscript{NTR} in both BT73 and BT206 increased the number of neurospheres >10\(\mu\)m. Values shown are the mean \(\pm\) SEM from three independent experiments. ** indicates \(P< 0.001\), and * indicates \(P< 0.01\). Analysis was performed with Student t-test.
A

**BT73**
- BT73 Isotype
- BT73 control
- BT73 p75NTR

**BT206**
- BT206 Isotype
- BT206 control
- BT206 p75NTR

B

**BT73**
- **CD15**: ns
- **CD133**: ***

**BT206**
- **CD15**: ***
- **CD133**: ***
Figure 3.14 p75NTR overexpression in patient-derived BTICs increased the expression of stem cell markers CD15 and CD133

(A) Flow cytometry analysis of CD15 and CD133 expression in BT73 control and BT73p75NTR cells or BT206 control and BT206p75NTR cells. Histograms of CD15, but not CD133, of BT73p75NTR cells indicate that the percentage of cells expressing CD15 is greater than the percentage of cells expressing CD15 in BT73 control cells (upper panels). Histograms of CD15 and CD133 of BT206p75NTR cells indicate the percentage of cells expressing CD15 and CD133 is greater than percentage of cells expressing CD15 and CD133 in BT206 control cells (lower panels). Isotype controls were used to exclude non-specific background signals of used antibodies.

(B) Quantification of the percentage of cells expressing CD15 and CD133 in BT73 control and BT73p75NTR (left panel) or in BT206 control and BT206p75NTR (right panel). Overexpression of p75NTR increased the expression of CD15 in BT73p75NTR and BT206p75NTR cells compared to BT73 control and BT206 control cells respectively. Overexpression of p75NTR increased the expression of CD133 in BT206p75NTR cells compared to BT206 control cells and no difference was found in the expression of CD133 between BT73 control and BT73p75NTR cells. Values shown are the mean ± SEM from three independent experiments. NS indicates no statistical significance; *** indicates $P<0.0001$. Analysis was performed with 2-way ANOVA with Bonferroni’s multiple comparisons test.
3.3.2 Expression of p75NTR increases invasion of genetically distinct BTICs in vitro and in vivo

Previously, our laboratory showed that p75NTR induced glioma invasion of genetically distinct glioma cell lines in vitro and in vivo (Ahn et al., 2016; A. L. Johnston et al., 2007; L. Wang et al., 2008). To test whether p75NTR is also able to induce invasion of genetically distinct BTICs, BT73p75NTR and BT206p75NTR and their controls were assessed for their invasive ability using a transwell invasion assay. Consistent with our previous findings, p75NTR induced invasion of both BT73p75NTR and BT206p75NTR compared to their control counterparts (Figure 3.15.A). To determine whether the expression of p75NTR is able to induce BTICs invasion in vivo, we implanted BT73p75NTR, BT206p75NTR and their controls into the brains of SCID mice. Three weeks later, animals were sacrificed and their brains were isolated and processed for histopathological analysis. Immunofluorescence staining of brain sections prepared from these mice showed high p75NTR expression on BT73p75NTR and BT206p75NTR compared to their controls (Figure 3.15.B). The whole brain stitched images showed that ectopic expression of p75NTR in both BT73 and BT206 cells resulted in formation of invasive tumors with some cells invading the corpus callosum (Figure 3.15.C). Furthermore, the control BT73 and BT206 cells formed tumors with some invasive cells migrating outside the tumor mass. Immunostaining analysis showed that some of these invasive cells expressed high endogenous p75NTR levels (Figure 3.15.D), confirming the role of p75NTR in inducing glioma cell invasion. To determine if ectopic p75NTR in BTIC induces more proliferation as we observed in vitro, mice bearing brain tumors formed by p75NTR-expressing BT73p75NTR and BT206p75NTR cells or by their controls, BT73 and BT206 cells, were injected with BrdU 24 hours before sacrificing to identify the percentage of proliferative cells using anti-BrdU specific antibody. Immunofluorescent analysis of the percentage of BrdU positive cells in the brain section of mice bearing tumours formed by BT73 control or BT73p75NTR cells, or BT206
control and BT206p75^{NTR} cells showed no marked difference in the percentage of BrdU positive cells as shown in Figure 3.16.A and quantified in Figure 3.16.B. Furthermore, no significant difference was seen in the survival of mice bearing tumours formed by BT73 control or BT73p75^{NTR} cells, or BT206 control and BT206p75^{NTR} cells (Figure 3.16.C). These results suggest that both BT73 and BT206 cell lines are highly proliferative and p75^{NTR} ectopic expression in these cells had no effects on their proliferation in vivo.
C

BT73 Control

BT73p75

BT206 Control

BT206p75

Human nucleolin

D

BT73 Control

Human nucleolin\_p75

BT206 Control
Figure 3.15. Ectopic expression of \( p75^{\text{NTR}} \) induced patient-derived BTICs invasion in vitro and in vivo

(A) Transwell invasion assay of BT73 control and BT73p75\(^{\text{NTR}} \) cells (left panels) or BT206 control and BT206p75\(^{\text{NTR}} \) cells (right panels). Overexpression of \( p75^{\text{NTR}} \) in BT73 and BT206 induced their invasion as seen in images (upper panels) and quantified in (lower panels). Values shown are the mean ± SEM from three independent experiments. *** indicates \( P < 0.0001 \). Analysis was performed with Student t-test. (B) Immunofluorescent analysis of \( p75^{\text{NTR}} \) (green) in brain sections of mice bearing brain tumors formed by BT73 control or BT73p75\(^{\text{NTR}} \) cells (left panels) or BT206 control or BT206p75\(^{\text{NTR}} \) cells (right panels). Human-specific nucleolin antibody (red) was used to detect the human tumor cells. Both BT73p75\(^{\text{NTR}} \) and BT206p75\(^{\text{NTR}} \) cells have stronger \( p75^{\text{NTR}} \) expression compared to BT73 and BT206 control cells in these brain sections. Images were acquired by IN Cell Analyzer 6000 using 40X objective. (C) Immunofluorescent analysis of human nucleolin (red) to detect the human tumor cells in brain sections of mice bearing tumors formed by BT73 control or BT73p75\(^{\text{NTR}} \) cells (upper panels) or BT206 control or BT206p75\(^{\text{NTR}} \) cells (lower panels). Ectopic expression of \( p75^{\text{NTR}} \) in BT73 and BT206 induced the formation of invasive tumors compared to BT73 control and BT206 control cells that formed tumors with less invasive edges. Images were acquired by an IN Cell Analyzer 6000 with 4x objective and stitched using IN Cell Analyzer developer software. (D) Immunofluorescent analysis of \( p75^{\text{NTR}} \) (green) in brain sections of mice bearing brain tumors formed by BT73 control (left panels) or BT206 control cells (right panels) shows that invasive cells in these tumors express \( p75^{\text{NTR}} \) (green) compared to other cells in these tumors. Human-specific nucleolin antibody (red) was used to detect the human tumor cells. Images were acquired by an IN Cell Analyzer 6000 with 4x objective and stitched using IN Cell Analyzer developer software.
A

B

C

Brdu
Human nucleolin
merge

control

BT73

p75_{NTR}

Brdu
Human nucleolin
merge

control

BT206

p75_{NTR}

BT73

BT206

ns

ns

ns

Percent survival

Day

Percent survival

Day

BT73 control

BT73p75

BT206 control

BT206p75

ns

ns
Figure 3.16 p75NTR ectopic expression in patient derived BTIC had no effect on their proliferation in vivo

(A) Immunofluorescence images of Bromodeoxyuridine (BrdU) positive cells (green) in whole brain sections from animals implanted with BT73 control and BT73p75NTR cells (upper panels) or BT206 control and BT206p75NTR cells (lower panels). BrdU was injected into the animals 24 hours before sacrificing them. Brain sections were stained with anti-BrdU (green) and a human-specific nucleolin antibody (red) to detect the human tumor cells. Images were acquired by an IN Cell Analyzer 6000 with 40X objective. (B) Quantification of BrdU positive tumor cells in whole brain sections from mice implanted with BT73 control and BT73p75NTR cells (upper panel) or BT206 control and BT206p75NTR cells (right panel). Values shown are the mean ± SEM from three independent experiments. NS indicates no statistical difference; analysis was performed with Student t-test. (C) Kaplan-Meier survival curves of mice implanted with BT73 control and BT73p75NTR cells (left panel) or BT206 control and BT206p75NTR cells (right panel). No significant difference was found in the survival of mice implanted with BT73 control and BT73p75NTR cells (left panel) or BT206 control and BT206p75NTR cells (right panel); (n= 6 mice in each group).
3.3 Discussion

GBM is a highly aggressive tumor characterized by resistance to current chemotherapeutic and radiotherapy and correlated with poor patient prognosis due to frequent recurrence. These GBM features are attributed to the extensive heterogeneous nature of this tumor and the presence of self-renewing, tumorigenic BTICs that contribute to tumor initiation, progression and frequent relapse (Wilson, Karajannis, & Harter, 2014). Targeting these cells may provide therapeutic benefits, and sensitize GBM tumors to the current treatment regimens and extend survival of GBM patients. Here we demonstrated that p75NTR is variably expressed on genetically different GBM patient-derived BTICs. Targeting p75NTR on these cells dramatically inhibited their sphere-forming capacity and proliferation abilities, induced their differentiation and cellular death, and most importantly, increased the survival of mice bearing brain tumors generated by these cells significantly.

In recent years, there has been growing evidence of the importance of a p75NTR signaling axis in maintaining cancer stem cells in different cancers (reviewed in Chopin, Lagadec, Toillon, & Le Bourhis, 2016). Here we found that in p75NTR loss of function studies in BTICs there was a significant reduction of sphere-forming capacity of these cells and decreased expression level of stem and progenitor cell markers including Nestin, SOX2, Olig2, and CD44. In contrast, in p75NTR gain of function studies in BTICs that lack endogenous p75NTR expression there was a significant increase in the sphere-forming capacity of the cells and increased expression of stem cell markers CD133 and CD15, suggesting an essential role of p75NTR in the maintenance of stemness status of BTICs. Consistent with these findings, p75NTR was identified as a stem cell marker of human melanoma-initiating cells, and melanoma patient-derived p75NTR-positive but not p75NTR-negative cells had a high self-renewal capacity and were able to form tumors when transplanted into nude...
or non-obese diabetic/severe combined immunodeficient mice (A. D. Boiko et al., 2010). Importantly, p75NTR-positive cells were essential for continuous melanoma growth, as long-term passaging and expansion of tumors were dependent on the presence of a p75NTR-positive cell fraction in the tumor (A. D. Boiko et al., 2010; Civenni et al., 2011). In breast cancer stem cells, p75NTR was also found to increase self-renewal ability through upregulating the expression of stem cell pluripotency factors including SOX2, MYC, OCT4 and NANOG, and targeting p75NTR by siRNA reduced the expression level of these stem cell markers and inhibited the self-renewal capacity of these cells (Tomellini et al., 2015). Additionally, p75NTR was expressed on a subpopulation of medulloblastoma cells that were characterized by higher self-renewal capacity (Morrison et al., 2013). Collectively, all these findings suggest that p75NTR functions as a cancer stem cell marker in various cancers and contributes to the self-renewal ability of these cells.

The main characteristic of both normal and cancer stem and progenitor cells is their ability to self-renew and produce differentiated progeny. The balance between these processes is achieved through regulated asymmetric divisions, which are necessary to generate cellular diversity. Disruption of this balance may lead to a premature depletion of the stem/progenitor cell pool, or abnormal growth (Gomez-Lopez et al., 2014). Numb has been found to play a major role in regulating asymmetric cell divisions of mouse cerebral cortical stem cells, neuroblasts, and GBM stem cells (Lathia et al., 2011; Shen et al., 2002). Numb is a fate-determining molecule that induces the differentiation of neural stem cells through its ability to antagonize notch and hedgehog signaling pathways (Di Marcotullio et al., 2006; McGill, Dho, Weinmaster, & McGlade, 2009). Here we found p75NTR knockdown in BTICs changed their symmetry and asymmetry cell division balance and increased the percentage of Numb-expressing daughter cells, suggesting that p75NTR not only stimulates self-renewal ability of BTICs but also maintains the balance of stem cell pools.
Targeting $p75^{NTR}$ on these cells induced their division towards differentiated progenies. In agreement with these findings, NGF and proNGF treatment of $p75^{NTR}$-expressing breast cancer stem cells decreased Numb expression in daughter cells and increased symmetric cell divisions to maintain the proportion of self-renewing breast cancer stem cells (Tomellini et al., 2015). Taken together, these observations suggest that $p75^{NTR}$ mediates the enlargement of the cancer stem cell compartment in both GBM and breast cancer.

The balance between cell survival and cell death in an organism is key to maintaining normal tissue homeostasis. Malignant tumors can arise as a result of many abnormal changes in the biological processes of cells, one of which is their acquired ability to evade cell death in the presence of various types of stresses leading to their prolonged survival beyond normal limits (Hanahan & Weinberg, 2011). Here we found that $p75^{NTR}$ knockdown in BTICs induced their cell cycle arrest, increased their cell death and decreased tumor formation in vivo. In head and neck squamous cell carcinoma, $p75^{NTR}$ was expressed on the most tumorigenic cells and loss of $p75^{NTR}$ function resulted in a block of the G2-M phase of the cell cycle and markedly decreased the capacity of these cells to initiate tumor formation in vivo (Murillo-Sauca et al., 2014). In addition, $p75^{NTR}$ expression was negatively correlated with human hypopharyngeal cancer (HPC) prognosis and $p75^{NTR}$ knockdown in HPC cell lines induced their cell-cycle arrest in G0 and suppressed ERK phosphorylation and the cells’ ability to form tumors in vivo (Mochizuki et al., 2016). In breast cancer cells, $p75^{NTR}$ acted as an anti-apoptotic molecule and promoted cell survival through binding to the neurotrophin NGF and activating the NF-$\kappa$B pathway (Descamps et al., 2001). In breast cancer xenograft models, $p75^{NTR}$ promoted cell survival by increasing the expression of the inhibitor of apoptosis protein-1 (c-IAP1) and by decreasing (TNF-Related Apoptosis Inducing Ligand) TRAIL-induced cleavage of poly (ADP-ribose) polymerase PARP, pro-caspase 9 and
procaspase 3, and contributed to tumor resistance to drugs (Verbeke et al., 2010). Gene set enrichment analysis of melanoma cells that express p75<sup>NTR</sup> and have chemoresistant activities revealed a strong association between p75<sup>NTR</sup> expression and genes involved in the regulation of DNA repair genes. The knockdown of p75<sup>NTR</sup> in these cells decreased the expression of some DNA repair genes and partially increased sensitivity of these cells to chemotherapeutic agents (Redmer et al., 2017). In addition, p75<sup>NTR</sup> promotes melanoma cell survival and drives acquired resistance to the BRAF inhibitor vermurafenib, a highly effective treatment of melanoma. The expression of p75<sup>NTR</sup> was induced upon vermurafenib treatment through a stimulation of tumor necrosis factor-alpha (TNFα) secretion that leads to NF-κB signaling pathway activation. The silencing of p75<sup>NTR</sup> and as the inhibition of the TNFα/NF-κB pathway increased the sensitivity of vermurafenib in melanoma resistant cell lines (Lehraiki et al., 2015). Collectively, these findings suggest that p75<sup>NTR</sup> contributes to several cancers by increasing cancer cell proliferation and cell survival as well as promoting drug resistance in some cancers. On the contrary, p75<sup>NTR</sup> was found to act as a potential tumor suppressor in several cancers. For instance, p75<sup>NTR</sup> expression was absent or significantly decreased in a large cohort of gastric cancers compared with expression in the normal gastric mucosa. Ectopic expression of p75<sup>NTR</sup> in several gastric cancer cell lines dramatically attenuated cell proliferation and inhibited the cells’ ability to form tumors in animal models by inducing their cell cycle arrest (Jin et al., 2007). Similarly, ectopic expression of p75<sup>NTR</sup> in bladder and prostatic cancer cells was also found to inhibit proliferation, induce apoptosis and attenuate tumorigenic ability of these cells <i>in vivo</i> (Krygier & Djakiew, 2002; A. Tabassum, F. Khwaja, & D. Djkiew, 2003). Taken together, all the different roles of p75<sup>NTR</sup> in various cancers suggest that the effect of p75<sup>NTR</sup> activation in cancer is cell type-specific and must be independently determined for each cell system studied. The various roles of p75<sup>NTR</sup> in different
cancers can likely be explained by the presence of distinct $p75^{\text{NTR}}$ co-receptors. For instance, opposite to the role of $p75^{\text{NTR}}$ in prostate cancer, Trk receptors play a major role in mediating proliferation of prostate cancer cells and suppression of Trk receptors led to inhibition of prostate cancer cell proliferation (Melck et al., 2000).

In agreement with our previous findings that $p75^{\text{NTR}}$ is a main regulator of glioma invasion (Ahn et al., 2016; A. L. Johnston et al., 2007; L. Wang et al., 2008), we found that the ectopic expression of $p75^{\text{NTR}}$ in BTICs lacking endogenous $p75^{\text{NTR}}$ expression induced their invasion both *in vitro* and *in vivo*. The correlation between $p75^{\text{NTR}}$ and increased invasive ability of cancer cells has been identified in other tumors. In melanoma, $p75^{\text{NTR}}$ was found to correlate with tumor advanced stages and associate with invasive potential of melanoma brain metastasis, and binding of NGF or pro-NGF to $p75^{\text{NTR}}$ on melanoma cells increased their migration dramatically (Denkins et al., 2004; Shonukan, Bagayogo, McCrea, Chao, & Hempstead, 2003). Recently, $p75^{\text{NTR}}$ was found to be essential for migration and survival of renal cell carcinoma because $p75^{\text{NTR}}$ silencing inhibited the growth and migration of the cancer cells (De la Cruz-Morcillo et al., 2016). The concept of $p75^{\text{NTR}}$ as a key regulator of migration and invasion is not limited to the cancer cells. Neural crest stem cells, a migratory cell population that differentiate to give rise during embryonic and adult development to a wide variety of cell and tissue types, including bones, cartilages, connective tissues, pigment and endocrine cells as well as neurons and glia amongst many others, express high levels of $p75^{\text{NTR}}$ even before they commit to any cell differentiation lineage (Dupin & Sommer, 2012; Stemple & Anderson, 1992). Furthermore, the examination of $p75^{\text{NTR}}$ knockout mouse embryos revealed that the migration of Schwann cells from the dorsal root ganglia was significantly decreased (Bentley & Lee, 2000). Collectively, these observations suggest that $p75^{\text{NTR}}$ is an important mediator of migration and invasion of cells in both in normal and
pathological conditions, however, molecular mechanisms underlying p75\textsuperscript{NTR}-mediated invasion and migration are largely cell-type specific and must be independently determined for each cellular context.

In conclusion, it appears that p75\textsuperscript{NTR} contributes to glioma progression through maintaining the sphere-forming capacity, proliferation, invasion and potential tumorigenic ability of GBM patient-derived BTICs, a cell population that has been shown to be involved in chemo- and radiotherapy resistance and disease relapse.
Chapter Four: Cell non-autonomous roles of the neurotrophin receptor \( p75^{NTR} \) in glioma invasion

4.1 Introduction

4.1.1 Rationale

One of the major hallmarks of malignant gliomas is their highly invasive nature. The major obstacle to cure glioma patients is the high capacity of glioma cells to invade and migrate throughout the surrounding brain tissue, which enables tumors to escape complete surgical resection and radio-and chemotherapy. The mechanism by which glioma cells invade into and throughout the brain is not completely understood, and investigating and understanding this mechanism at the molecular level may lead to the development of better therapeutics for glioma patients. While many studies have reported the cell autonomous role of several classical invasion-related genes, including different proteases and adhesion molecules, by investigating the effects of loss and gain of function of these genes on the invasive phenotypes mostly \textit{in vitro} and in some cases \textit{in vivo} (reviewed in (Mehta & Lo Cascio, 2018), very few studies have actually assessed whether cell non-autonomous mechanisms of these molecules in glioma invasion exist.

Tumor-derived extracellular vesicles (EVs) have emerged as important means of intercellular communication by transferring signaling proteins and genetic information to cells in their immediate vicinity and at distant locations. Several biological functions are attributed to EVs in cancer including invasion, migration, proliferation, metastasis, angiogenesis, immune system modulation and drug resistance (Simons & Raposo, 2009). In the context of glioma, glioma-derived EVs are likely to represent one of the mechanisms by which glioma cells change the microenvironment and make it permissive for invasion and growth.

Our laboratory previously identified \( p75^{NTR} \) as a central regulator of glioma invasion (A. L. Johnston et al., 2007). Ectopic expression of \( p75^{NTR} \) in genetically distinct non-invasive glioma
enhanced their invasive behaviour dramatically both *in vitro* and *in vivo*, and robust expression of p75\textsuperscript{NTR} was frequently detected in highly invasive GBM specimens (Ahn et al., 2016; A. L. Johnston et al., 2007; L. Wang et al., 2008). Furthermore, p75\textsuperscript{NTR}-mediated glioma invasion was shown to occur through a neurotrophin-dependent proteolytic process of the receptor that could be inhibited with clinically-relevant \(\gamma\)-secretase inhibitors (L. Wang et al., 2008). Collectively, these findings demonstrate the cell-autonomous role of p75\textsuperscript{NTR} in glioma invasion; however, the cell non-autonomous role of p75\textsuperscript{NTR} in different cancers generally, and in glioma specifically, has not been addressed. Therefore, the focus of this chapter was to determine whether p75\textsuperscript{NTR} is transported by glioma derived-EVs and whether p75\textsuperscript{NTR}-containing EVs contribute to the invasive behaviour of glioma cells.

### 4.1.2 Hypothesis

Our hypothesis is that p75\textsuperscript{NTR} contributes in part to the invasive behavior of GBM by releasing p75\textsuperscript{NTR}-containing EVs that act as a regulator of glioma infiltrative behavior.

### 4.1.3 Objectives

To address this hypothesis, three main objectives were outlined. The first was to determine whether p75\textsuperscript{NTR} is released in glioma-derived EVs. The second objective was to determine if the proteolytic processing of p75\textsuperscript{NTR} is required for p75\textsuperscript{NTR} to be loaded in EVs. The third objective was to determine the role p75\textsuperscript{NTR}-containing EVs in glioma invasion.
4.2 Results

4.2.1 Secretome of p75NTR expressing cells induces glioma invasion

The ectopic expression of p75NTR in genetically distinct non-invasive glioma cell lines (U87 and U251N) was shown to induce their invasion and migration in vitro and in vivo (A. L. Johnston et al., 2007), suggesting that p75NTR mediates glioma cell invasion in a cell autonomous mechanism. Cancer cells secrete numerous factors including growth factors, cytokines, adhesion molecules, shed receptors and proteases that reflects the functionality of these cells at a given time point. These secreted factors, collectively known as cancer cell secretome, have been implicated in activating paracrine signaling on neighboring cells to induce invasion, migration, proliferation, angiogenesis, evasion of apoptosis, and resistance to anti-proliferative signals and chemotherapy (Karagiannis, Pavlou, & Diamandis, 2010). The cancer cell secretome can be characterized in the medium where these cells are cultured; therefore, the medium is known as conditioned medium (CM). To investigate if p75NTR contributes to glioma invasive behavior via a cell non-autonomous mechanism, we examined whether CM of p75NTR-expressing cells (U87-p75NTR and U251-p75NTR) is able to induce the invasion of non-invasive glioma cells (U87-pcDNA and U251-pcDNA). CM of invasive p75NTR-expressing cells (U87-p75NTR and U251-p75NTR) was incubated with the non-invasive cells (U87-pcDNA and U251-pcDNA) in the upper chamber of transwell invasion inserts as shown in Figure 4.1.A. The CM from both U87-p75NTR and U251-p75NTR cells induced invasion of U87-pcDNA and U251-pcDNA cells (Figures 4.1.B and C), suggesting that the secretome of p75NTR-expressing glioma cells includes certain molecules that contribute to glioma cell invasion. In eukaryotic cells, soluble proteins are released into the extracellular space either by exocytosis of secretory vesicles or by release of secretory/storage granules upon stimulation and activation of intracellular signaling pathways, including transportation of
synthesized proteins from endoplasmic reticulum (ER) to the Golgi apparatus and subsequently to the cell surface. This pathway has been identified as classical secretory pathway (Mellman & Warren, 2000; Walter, Gilmore, & Blobel, 1984). Proteins are also released from cells through non-classical secretory pathways. Certain proteins are imported into specific endosomal vesicles that are fused with plasma membrane through a process called endosomal recycling, resulting in the release of these proteins into the extracellular space (Rubartelli, Cozzolino, Talio, & Sitia, 1990). Other proteins are released into the extracellular space as a result of direct translocation across the plasma membrane using distinct transport systems (Mignatti, Morimoto, & Rifkin, 1992). Moreover, proteins can also be secreted through exosomes that are formed and released upon maturation of the endosomal pathway. This process starts by the internalization of activated receptors and the scaffolding proteins present therein, followed by internalization within the endosome forming a multi-vesicular body (MVB), that further matures to form the intraluminal vesicles (ILVs). Upon fusion of the MVB with the plasma membrane these vesicles are released into the extracellular space and referred to as exosomes (Johnstone, Adam, Hammond, Orr, & Turbide, 1987). To identify the molecule(s) in the secretome of p75NTR-expressing cells that induced glioma invasion, and if the secretome is secreted freely in the CM or packaged in secreted vesicles, CM was recruited for differential centrifugation steps that resulted in purified EV and EV-depleted CM. These purified products were used independently to examine their ability to induce glioma invasion. Treatment of non-invasive glioma cell U87-pcDNA or U251-pcDNA with purified EVs isolated from CM of p75NTR-expressing cells (U87-p75NTR or U251-p75NTR cells) resulted in a significant increase in glioma invasion, whereas there was no significant increase in the invasion of glioma cells treated with EV-depleted CM (Figures 4.2.A and C). These results suggest that the secretome of p75NTR-expressing cells, particularly secreted EVs, contains specific
molecules that enhance glioma invasion. Our lab previously showed that p75NTR is a central mediator of glioma invasion (Ahn et al., 2016; Alshehri et al., 2017; A. L. Johnston et al., 2007; L. Wang et al., 2008). In sympathetic neurons and rat PC12 cells, p75NTR evades the endolysosomal route, favouring MVB specialized for exosomal release (Escudero et al., 2014). Taken together, these findings suggest that p75NTR might be released from glioma cells in EVs that contribute to glioma invasion. To test this hypothesis and to assess the abundance, if any, of secreted p75NTR, CM of U87p75NTR was used for immunoblotting analysis. The analysis showed that p75NTR is detectable in the CM of U87p75NTR and U251-p75NTR cells but not in the CM of U87-pcDNA or U251-pcDNA cells (Figures 4.2.B and D). The mechanisms that trigger EV generation by cancer cells are unknown, but loss of tumor suppressor genes and expression of several oncogenes that enhance invasion and tumor growth of cancer cells have been associated with extensive secretion of EVs (Al-Nedawi et al., 2008; X. Yu et al., 2006). For instance, the loss of p53, a tumor suppressor that is highly mutated in glioma, influences the secretion of high amounts of tissue factor-containing EVs into the blood of tumor-bearing mice (J. L. Yu et al., 2005). Furthermore, EGFRvIII expression in indolent glioma cells induces the formation and release of EGFRvIII-containing EVs (Al-Nedawi et al., 2008). These findings prompted us to investigate if p75NTR expression in glioma cells correlates with increased EV secretion. Interestingly, we observed a dramatic increase of EV-like structures on the cell surface of U87p75NTR and U251p75NTR glioma cells compared to their non-p75NTR expressing cells, U87-pcDNA and U251-pcDNA cells, as detected by scanning electron microscopy (Figures 4.3.A and B). This increase was accompanied by a corresponding increase in the number of EVs released into the medium of the respective cell lines as quantified by Nanosight LM10 instrument (Figure 4.4.A). These observations suggest that p75NTR expression induces the production and release of EVs. Transmission electron microscopy
(TEM) is considered the gold standard method for characterizing the morphology of EVs (van der Pol et al., 2010). EVs of both U87-pcDNA and U87-p75NTR were isolated by differential centrifugation, processed and immediately visualized using TEM. EVs isolated from both U87-pcDNA and U87-p75NTR exhibited a round morphology and uniform, unimodal distribution in size that was consistent with known morphological characteristics of EVs (Figure 4.4.B). These results suggest that glioma cells release EVs that exhibit the known morphological shapes and p75NTR expression may not affect the morphological structure of these vesicles.
Figure 4.1 Secretome of p75NTR-expressing cells induces glioma invasion

(A) A schematic of the transwell invasion assay. Non-invasive glioma cells were incubated with conditioned medium (CM) of invasive, p75NTR-expressing cells in the upper chamber of the transwell insert. Five hours after incubating cells at 37°C, cells were fixed and stained with crystal violet. Cells that invaded through the collagen I layer to the trans-side of the membrane were quantified using bright field microscopy. (B, C) Quantification of the transwell invasion assay shows that incubation of non-invasive U87-pcDNA and U251-pcDNA with CM from invasive, p75NTR-expressing U87-p75NTR and U251-p75NTR cells increased the cells’ invasion significantly. Values show the mean ± SEM from three independent experiments; *** indicates $P < 0.0001$. (B) or $P < 0.0001$ vs U251-pcDNA (C). Analysis was performed with one-way ANOVA with Neuman-Keuls post-test.
A

[Bar chart showing invasive cell counts for different groups: U87-pcDNA (non-invasive), U87-pcDNA + EV-depleted, U87-pcDNA + EVs isolated from CM of U87-p75+ cells, U87-p75+ (invasive).]

*** ns

B

[Western blot images for Cell Lysate and Conditioned medium showing bands at 75kDa, 25kDa, and 19kDa for p75WT, CTF, and ICD, respectively.]

C

[Bar chart showing invasive cell counts for different groups: U251-pcDNA (non-invasive), U251-pcDNA + EV-depleted, U251-pcDNA + EVs isolated from CM of U251-p75+ cells, U251-p75+ (invasive).]

*** ** ns

D

[Western blot images for Cell Lysate and Conditioned medium showing bands at 75kDa, 25kDa, and 19kDa for p75WT, CTF, and ICD, respectively.]
Figure 4.2 EVs of p75NTR-expressing cells induce glioma invasion

(A) Quantification of transwell invasion assay shows that EVs of U87-p75NTR cells are able to induce the invasion of U87-pcDNA significantly compared to untreated U87-pcDNA cells or U87-pcDNA cells treated with EV-depleted U87-p75NTR conditioned medium (CM). U87-p75NTR cells were used as a positive control. Values show the mean ± SEM from three independent experiments; ns indicates no statistical significance; *** indicates $P < 0.0001$, ** indicates $P < 0.001$. Analysis was performed with one-way ANOVA with Neuman-Keuls post-test. (B) Western blot analysis shows that p75NTR is secreted in the CM of U87-p75NTR cells but not in CM of U87-pcDNA cells. Cell lysates of U87-pcDNA and U87-p75NTR were used as negative and positive controls. (C) Quantification of transwell invasion assay shows that EVs of U251-p75NTR cells are able to induce the invasion of U251-pcDNA significantly compared to untreated U87-pcDNA cells or U87-pcDNA cells treated with EVs-depleted U251-p75NTR CM. U251-p75NTR cells were used as a positive control. (D) Western blot analysis shows that p75NTR is secreted in the CM of U251-p75NTR cells but not in the CM of U251-pcDNA cells. Cell lysate of U251-pcDNA and U251-p75NTR were used as negative and positive controls, respectively. Values show the mean ± SEM from three independent experiments; ns indicates no statistical significance; *** indicates $P < 0.0001$; ** indicates $P < 0.001$. Analysis was performed with one-way ANOVA with Neuman-Keuls post-test.
A
SEM image of U87-pcDNA cells (10K x)  
SEM image of U87-p75^NTR cells (10K x)

B
SEM image of U251-pcDNA cells (10K x)  
SEM image of U251-p75^NTR cells (10K x)
Figure 4.3 $p75^{NTR}$ induces the formation of EVs-like structure

Scanning electron microscopy images show that $p75^{NTR}$ expression induces the formation of vesicular membrane protrusions in U87-$p75^{NTR}$ cells compared to U87-pcDNA (A) and U251-$p75^{NTR}$ compared to U251-pcDNA (B).
TEM image of U87-pcDNA EVs

TEM image of U87-p75NTR EVs
Figure 4.4 \textit{p75}^{NTR} induces EVs secretion from human glioma cells

(A) Quantification of EVs released from 10 million cells of U87-pcDNA and U87-p75\textit{NTR}. EVs were isolated from CM and quantified using the NanoSight LM10 instrument. Ectopic expression of \textit{p75}^{NTR} in human glioma cells (U87-p75\textit{NTR}) increased EV secretion significantly compared to that in U87-pcDNA cells. Values shown are the mean ± SEM from three independent experiments; * indicates \( P = 0.0247 \); analysis was performed with unpaired two-tailed test. (B) Transmission electron microscope (TEM) images of EVs isolated from CM of U87-pcDNA and U87-p75\textit{NTR} show characteristic spheroid morphology of these vesicles. Scale bar: 100 nm in the upper panel and 50 nm in the lower panel.
4.2.2 p75\textsuperscript{NTR} proteolytic process can be detected in EVs but it is not required for p75\textsuperscript{NTR} to be loaded on EVs

p75\textsuperscript{NTR} is a substrate for sequential $\alpha$ and $\gamma$ secretase–mediated intramembrane proteolysis generating 24 kDa CTF and 19 kDa ICD fragments, respectively, and the generation of these fragments are required for neuronal cell death (Underwood, Reid, May, Bartlett, & Coulson, 2008), cell survival (Kommaddi, Thomas, Ceni, Daigneault, & Barker, 2011) and glioma cell invasion (L. Wang et al., 2008). A schematic figure illustrating this process is shown in Figure 4.5.A. We sought to determine whether p75\textsuperscript{NTR} is packaged in EVs and whether intramembrane proteolysis of p75\textsuperscript{NTR} occurs in EVs released from glioma cells. To do this, we assessed if the full-length of p75\textsuperscript{NTR} is detectable in EVs and whether the generation of 24-kDa CTF and 19-kDa ICD occurs in EVs released by U87-p75\textsuperscript{NTR} and U251-p75\textsuperscript{NTR} glioma cells and a panel of patient-derived BTICs. EVs isolated by differential centrifugation were digested in a lysis buffer and analyzed by western blots using a p75\textsuperscript{NTR} cytoplasmic-specific antibody that detected the full-length p75\textsuperscript{NTR} protein and the p75\textsuperscript{NTR} fragments migrating at 24 and 19 kDa, respectively. Western blot analysis showed that in addition to the 75-kDa full-length p75\textsuperscript{NTR} protein, 24-kDa and 19-kDa fragments were present in EVs isolated from p75\textsuperscript{NTR}-expressing glioma cells (U87-p75\textsuperscript{NTR} and U251-p75\textsuperscript{NTR}) (Figures 4.5.B and C) as well as in EVs isolated from p75\textsuperscript{NTR}-expressing patient-derived BTICs (Figure 4.5.D). Hence, p75\textsuperscript{NTR} processing occurs in EVs derived from endogenously p75\textsuperscript{NTR}-expressing patient-derived BTICs. This suggested the possibility that p75\textsuperscript{NTR} proteolytic processing is required for p75\textsuperscript{NTR} to be packaged in EVs. To address this possibility, U87-p75\textsuperscript{NTR} cells were treated with increasing concentrations (2, 4, 6 and 8$\mu$M of Compound X (Calbiochem), a specific inhibitor of $\gamma$-secretase, for 48 hours. After 48 hours, cells and their derived EVs were isolated, digested in lysis buffer and analyzed by western blots. Western blot analysis of p75\textsuperscript{NTR} revealed that in the presence of the $\gamma$-secretase inhibitor, U87-p75\textsuperscript{NTR} cells and their derived EVs
have an accumulation of the 24-kDa fragment without subsequent cleavage to the 19-kDa ICD, consistent with the release of the ICD of p75NTR by γ-secretase. (Figure 4.6.A). These results demonstrate that regulated intramembrane proteolysis of p75NTR is a global event occurring in highly invasive p75NTR-positive human glioma cells and their derived EVs. Moreover, p75NTR proteolytic processing in p75NTR-expressing cells is reflected in EVs derived from these cells. Previously, we have shown that proteolytic processing of p75NTR is a requirement for the highly invasive behavior of p75NTR-positive malignant glioma, and inhibiting this proteolytic process with the γ-secretase inhibitor, Compound X, significantly abrogated p75NTR-induced glioma invasion both in vitro and in vivo (L. Wang et al., 2008). To test whether proteolytic processed p75NTR-containing EVs have a functional role in glioma invasion, we analyzed invasive ability of non-invasive glioma cells (U87-pcDNA) incubated with EVs isolated from p75NTR-expressing cells grown in the absence and presence of Compound X (4μM). Interestingly, proteolytic processed p75NTR-containing EVs significantly induced the invasive ability of non–invasive glioma cells (U87-pcDNA and U251-pcDNA) to comparable invasive levels of U87-p75NTR and U251-p75NTR cells, while EVs that contain inhibited proteolytic processing of p75NTR had no effect on invasion of glioma cells (Figures 4.6.B and C). These results suggest that proteolytic processing of p75NTR is essential for p75NTR-mediated glioma cell invasion and also for p57NTR-containing EVs to induce invasion of non-invasive glioma cells.
A

Protease → α-secretase → γ-secretase

γ-secretase inhibitors

ECD: Extracellular Domain
CTF: C-terminal Fragment
ICD: Intracellular Domain

B

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75kDa
24kDa
19kDa
ECD
CTF
ICD

Invasive Behavior
**Figure 4.5** p75\textsuperscript{NTR} proteolytic processing occurs in EVs of p75\textsuperscript{NTR}-glioma cells

(A) A schematic figure of proteolytic processing of p75\textsuperscript{NTR}. Initially, p75\textsuperscript{NTR} is cleaved by a wide range of metalloproteinases such as α-secretase to shed the extracellular domain (ECD), leaving an unstable membrane-bound C-terminal fragment (CTF) that is further cleaved by a γ-secretase to release an intracellular domain (ICD) which is essential for p75\textsuperscript{NTR}-mediated glioma invasion, a process that can be inhibited using γ-secretase inhibitors. (B, C) Western blot analysis shows that U87-p75\textsuperscript{NTR} and U251-p75\textsuperscript{NTR} and their derived EVs are positive for full-length p75\textsuperscript{NTR} and migrating CTF and ICD fragments. U87-pcDNA and U251-pcDNA and their derived EVs were used as negative controls. (D) Western blot analysis shows that EVs isolated from patient-derived BTICs also express the full-length p75\textsuperscript{NTR} and the CTF and ICD fragments. Tumor susceptibility gene 101 (TSG 101) and Flotillin-1 were used as markers for EVs.
Figure 4.6 proteolytic processed p75NTR-containing EVs induced glioma cell invasion

(A) The highly invasive glioma cell line U87-p75NTR was treated with increasing concentrations of Compound X (4, 6, 8µM) for 48 hours. Cells and their derived EVs were isolated, digested and analyzed by Western blot. The western blot for p75NTR was probed with an antibody specific to the cytoplasmic domain of p75NTR that detects full-length (75 kDa), (CTF; 24 kDa), and (ICD; 19 kDa) peptides. The ICD is derived from the cleavage of the CTF, which is shown to visibly accumulate in the presence of a γ-secretase inhibitor in both U87-p75NTR and their derived EVs. (B) Transwell invasion assay of non-invasive U87-pcDNA glioma cells incubated with EVs isolated from U87-p75NTR cells in the presence or absence of 4 µM of Compound X. Cells were fixed and stained, and invasive cells were counted. U87-pcDNA, and U87-p75NTR cells were used as negative and positive controls, respectively. Proteolytic processed p75NTR-containing EVs significantly induced the invasive ability of non–invasive glioma cells (U87-pcDNA) to invasive levels comparable to U87-p75NTR cells. EVs that contain inhibited proteolytic processing of p75NTR had no effect on invasion of glioma cells. (C) Transwell invasion assay of non-invasive U251-pcDNA glioma cells incubated with EVs isolated from U251-p75NTR cells in the presence or absence of 4 µM of Compound X. Cells were fixed and stained, and invasive cells were counted. U251-pcDNA, and U251-p75NTR cells were used as negative and positive controls, respectively. Proteolytic processed p75NTR-containing EVs significantly induced the invasive ability of non–invasive glioma cells (U251-pcDNA) to comparable invasive levels of U251-p75NTR cells, whereas EVs that contain inhibited proteolytic processing of p75NTR had no effect on invasion of glioma cells. Values show the mean ± SEM from three independent experiments; ns indicates no statistical significance; *** indicates \( P < 0.0001 \). (B) or \( P < 0.0001 \) vs. U251-pcDNA (C); ** indicates \( P = 0.002 \); ns indicates no statistical significance. Analysis was performed with one-way ANOVA with Neuman-Keuls post-test.
4.2.3 EVs transfer p75NTR between cells to induce glioma invasion

It is well-established that γ-secretase has many substrates that may be involved in mediating glioma invasion; therefore, we decided to investigate the direct role of p75NTR-proteolytic processing on glioma cell invasion mediated by p75NTR-containing EVs (Kopan & Ilagan, 2004). To directly test the effect of p75NTR-proteolytic processing on p75NTR-containing EV-mediated glioma cell invasion, we investigated the role of EVs isolated from cells that express a cleavage-resistant chimera of p75NTR on glioma cell invasion. This p75NTR cleavage-resistant chimera was constructed by replacing the extracellular stalk domain of p75NTR (p75FasS) with equivalent domains from the Fas receptor. Both p75NTR and Fas receptors are members of the TNF receptor superfamily, and although they contain similar domains, Fas, unlike p75NTR, does not undergo RIP (L. Wang et al., 2008). A schematic figure of both p75NTR and p75FasS structures is shown in Figure 4.7.A. Expression of this cleavage-resistant chimera of p75NTR dramatically inhibited glioma invasion both in vitro and in vivo (L. Wang et al., 2008). To directly test the role of proteolytic processing of p75NTR in packaging p75NTR glioma-derived EVs, we evaluated the proteolytic processing of the cleavage-resistant chimeric proteins of p75NTR (p75FasS) in glioma cells and their derived EVs. Western blot analysis showed that only the full-length protein was detected for U87-p75FasS, and U251-p75FasS cells and their derived EVs, consistent with inhibition of the α-secretase cleavage (Figures 4.7.B and C). These results demonstrate that proteolytic processing of p75NTR is not required for p75NTR-packaging in glioma derived EVs. To test whether inhibition of p75NTR proteolytic processing has effects on the amount of EVs released by glioma cells, EVs were isolated from the CM medium of an equal number of U87-p75NTR and U87-p75FasS cells and from U251p75NTR grown in the presence or the absence of Compound X. Quantification of EVs isolated from these cells revealed no significant changes in the number of
EVs released either from U87-p75NTR and U87-p75FasS or U25-p75NTR cells grown in the presence of absence of Compound X (Figures 4.7.D and E). Taken together, these results suggest that inhibition of the p75NTR proteolytic process does not affect the packaging of p75NTR in EVs and the number of released EVs. To directly test the effect of p75NTR-proteolytic processing inhibition on p75NTR-containing EV-mediated glioma cells invasion, non-invasive U87-pcDNA or U251-pcDNA glioma cells were incubated with equal amounts of EVs isolated from either invasive cells that express the wild type p75NTR (U87-p75NTR or U251-p75NTR) or non-invasive cells that express the cleavage-resistant chimeric proteins of p75NTR (p75FasS) (U87-p75FasS or U251p75FasS) for 24 hours. The cells were then assessed for their invasive ability using a transwell invasion assay. EVs containing the wild-type p75NTR induced the invasive ability of both U87-pcDNA and U251-pcDNA compared to EVs containing the cleavage-resistant chimeric p75FasS (Figures 4.8.A and B). These results suggest that the proteolytic process of p75NTR is required for p75NTR-containing EVs to induce glioma invasion. To test the possibility that the wild-type p75NTR-containing EV-induced glioma invasion resulted from more uptake of the EVs by the non-invasive cells, U251-pcDNA, compared to EVs that do not contain p75NTR or contain the p75FasS, equal amounts of EVs that contain wild-type p75NTR or p75FasS were fluorescently labelled with a green-fluorescent lipophilic dye, DiI, which is incorporated in the outer membrane of EVs, and incubated with U251-pcDNA cells for 24 hours before imaging the cells under fluorescent microscopy. There was no significant difference in the uptake of EVs containing the wild-type p75NTR or p75FasS (Figure 4.8.C), providing evidence to support a role wild-type p75NTR-containing EVs in mediating glioma invasion. In glioma, EGFRvIII, a truncated and oncogenic form of the epidermal growth factor receptor, can be transferred between glioma cells through EVs to induce several oncogenic activities, including activation of transforming signaling
pathways (Akt and MAPK), changes in expression of EGFRvIII-regulated genes (VEGF, Bcl-xL, p27), morphological transformation and increases in anchorage-independent growth capacity (Al-Nedawi et al., 2008). To test whether p75NTR could be transferred in this manner from more invasive glioma cells to their non-invasive counterparts, p75NTR-negative U87 cells (U87-pcDNA) were incubated with equal amount of EVs isolated from glioma cells expressing the wild-type p75NTR(U87-p75NTR) or glioma cells expressing the p75NTR mutant form p75FasS (U87p75FasS) for 24 hours. Cells were lysed and western blots for p75NTR were performed. The analysis showed that U87 cells incubated with p75NTR-containing EVs expressed full-length p75NTR while cells incubated with p75NTRFasS-containing EVs expressed the p75FasS (Figure 4.9.A). To examine whether the de novo p75NTR expression in non-p75NTR expressing cells can occur in the glioma microenvironment through exchange of p75NTR-containing EVs, we mimicked this environment by using EVs isolated from patient derived-BTICs that endogenously express p75NTR and incubated these vesicles with non-p75NTR-expressing U87 glioma cells for 24 hours. Western blot analysis showed that incubation of BTIC-derived EVs with recipient cells resulted in detectable levels of p75NTR in these cells (Figure 4.9.B). Collectively, these observations suggest that p75NTR contributes to the invasive behaviour of glioma cells through a cell non-autonomous mechanism. Although the common technique of EV purification from CM is the differential centrifugation method, which typically consists of low-speed centrifugation to remove cells and large vesicles, and high-speed ultracentrifugation to pellet EVs (Thery et al., 2006), OptiPrep™ gradient isolation method has been found to provide the highest purification of EVs by removing contaminant impurities such as non-specific argonaute proteins (Van Deun et al., 2014). To confirm the findings that p75NTR-containing EV induction of glioma invasion is a result of the uptake of these vesicles and not to the presence of other possible contaminants, we isolated EVs from p75NTR-expressing
cells (U87-p75NTR and U251p75NTR), p75FasS cells (U87-p75FasS and U251p75FasS) and non-p75NTR-expressing cells (U87-pcDNA and U251-pcDNA) (Figure 4.10.A) and used them in a transwell invasion assay. Uptake of EVs isolated by OptiPrep™ gradient by non-invasive glioma cells U87-pcDNA resulted in a significant increase of invasive behaviour of cells treated with p75NTR-containing EVs but not p75FasS-containing EVs (Figure 4.10.B), confirming that p75NTR-containing EVs are major inducers of glioma invasion. It is well known that the microenvironment of tumors can change the biochemical characterization and function of cells. We have demonstrated in vitro that glioma cells including patient derived-BTICs released EVs that contain proteolytic processed p75NTR. We addressed whether this was also true for in vivo derived EVs. EVs were purified from serum of brain tumor-bearing mice (n = 6) generated from patient-derived BTICs that express p75NTR ectopically (BT206p75NTR), and from a control mice and analyzed by western blot. Western blot analysis for p75NTR showed that EVs isolated from serum of brain tumor-bearing mice have variable levels of p75NTR. Two mice showed a detectable proteolytic processed p75NTR in their EVs compared to the control mouse (Figure 4.11). Although it is not clear in this experiment if these vesicles derived from brain tumor cells, the results indicate that p75NTR is indeed packaged in EVs isolated form in vivo but its pathological role has yet to be determined.
Figure 4.7 Proteolytic processing of p75<sup>NTR</sup> is not required for it to be packaged in EVs

(A) Schematic diagram of the p75<sup>NTR</sup> constructs. Chimeric protein was created by replacing the extracellular stalk (S) domain of p75<sup>NTR</sup> with the equivalent domain from the Fas receptor. The protein was designated p75FasS. (B and C). Western blot analysis using a polyclonal p75<sup>NTR</sup> antibody to the intracellular domain of p75<sup>NTR</sup> confirmed the proper expression and processing of p75<sup>NTR</sup> construct (p75FasS) in both U87 human glioma cells and their derived EVs (B) and U251 human glioma cells and their derived EVs (C). (D) Quantification of EVs released from 5 million cells of U87-p75<sup>NTR</sup> or U87-p75FasS. EVs were isolated from conditioned medium (CM) and quantified using The NanoSight LM10 Instrument. No statistically significant difference was found. (E) Quantification of EVs released from 5 million cells of U251-p75<sup>NTR</sup> grown in the presence of 4 μM of the γ-secretase inhibitor, Compound X, (U251p75<sup>NTR</sup>+GSI) or in the absence of Compound X (U251-p75<sup>NTR</sup>). EVs were isolated from CM and quantified using The NanoSight LM10 Instrument. No statistically significant difference was found.
Figure 4.8 p75NTR-containing EVs induce glioma invasion

(A) Quantification of transwell invasion assay of non-invasive U87-pcDNA human glioma cells incubated with or without EVs isolated from either U87-pcDNA, U87-p75NTR or U87-p75FasS cells. Invasive cells were fixed, stained, and counted. EVs isolated from U87-p75NTR induced the invasive ability of non-invasive U87-pcDNA glioma cells significantly but no statistically significant difference in invasive ability of U87-pcDNA cells incubated with EVs isolated from U87-pcDNA or U87-p75FasS was found. Values shown are the mean ± SEM from three independent experiments; ns indicates no statistical significance; *** indicate $P < 0.001$. Analysis was performed by one-way ANOVA with Neuman-Keuls post-test. (B) Quantification of transwell invasion assay of non-invasive U251-pcDNA human glioma cells incubated with or without EVs isolated from either U251-pcDNA, U251-p75NTR or U251-p75FasS cells. Invasive cells were fixed, stained, and counted. EVs isolated from U87-p75NTR induced the invasive ability of non-invasive U251-pcDNA glioma cells significantly but no statistically significant difference in invasive ability of U251-pcDNA cells incubated with EVs isolated from U251-pcDNA or U251-p75FasS was found. Values shown are the mean ± SEM from three independent experiments; ns indicates no statistical significance, triple asterisks [***] indicate $P < 0.001$ vs. U251-pcDNA, one-way ANOVA with Neuman-Keuls post-test. (C) Human glioma cells, U251-pcDNA, were incubated with equal amount of fluorescently labelled EVs (50 µg/ml) isolated from U251-pcDNA, U251-p75NTR, U251-p75FasS or PBS as a negative control for 24 hours. Cells were washed three times, fixed, stained for actin and DAPI, and imaged using an IN Cell Analyzer 6000 (left panel). The EV uptake was determined based on the fluorescent intensity of at least 10 cells normalized to the U251-pcDNA control and the quantification represents the average of three independent experiments. No statistically significant difference in EVs uptake was observed.
Figure 4.9 EVs transfer p75NTR between glioma cells

(A and B) Western blot analysis of p75NTR transfer between glioma cells through EVs. EVs (50 µg/ml) isolated from U87-p75FasS or U87-p75NTR cells (A) or from BT25 or BT147 (B) were incubated with U87-pcDNA for 24 hours at 37 °C. Cells were washed three times with cold PBS, lysed and used for western blot analysis. Recipient cells of EVs-containing p75FasS are positive of p75FasS, similar to that present in EVs-containing p75FasS while U87-pcDNA incubated with EVs isolated from U87-p75NTR (A) or BT25 and BT147 (B) are positive for the p75NTR full-length.
Figure 4.10 Optiprep isolated p75<sup>NTR</sup> containing EVs induce glioma invasion

(A) Western blot analysis of the Optiprep gradient fractions 1–11 for p75<sup>NTR</sup> and the EV marker, flotillin-1, shows that EVs isolated from U87 and U251 human glioma cells are located in fractions 4–8. (B) Quantification of transwell invasion assay of non-invasive U87-pcDNA human glioma cells incubated with or without EVs isolated by Optiprep gradient method from either U87-pcDNA, U87-p75<sup>NTR</sup> or U87-p75FasS cells. Invasive cells were fixed, stained, and counted. EVs isolated from U87-p75<sup>NTR</sup> induced the invasive ability of non-invasive U87-pcDNA glioma cells significantly but no statistically significant difference in invasive ability of U87-pcDNA cells incubated with EVs isolated from U87-pcDNA or U87-p75FasS was found. Values shown are the mean ± SEM from three independent experiments; ns indicates no statistical significance; *** indicates $P < 0.001$. Analysis performed with one-way ANOVA with Neuman-Keuls post-test.
Figure 4.11 \( p75^{\text{NTR}} \) present in EVs isolated from serum of brain tumor-bearing mice

(A) Western blot analysis of \( p75^{\text{NTR}} \) protein levels in EVs isolated from serum of brain tumor-bearing mice generated by BT206p75\(^{\text{NTR}}\) (right panel). The full-length \( p75^{\text{NTR}} \) was detected in mouse 1, 3, 4, 5 and 6, whereas the \( p75^{\text{NTR}} \) cleavage fragments, CTF and ICD, were detected in mouse 3 and 6. \( p75^{\text{NTR}} \) protein was not detected in the EVs isolated from the control mouse. Alix was used as a marker for the EVs. Ponceau staining is shown as a loading control (left panel).
4.3 Discussion

GBMs are highly invasive tumors that associate with an extremely poor prognosis, even with all advances in surgical resection and the combination of chemo- and radiotherapy. The invasive behaviour of GBM cells is a main cause of the failure of radical surgical resection of the primary tumor mass, and invasive glioma cells invariably infiltrate within the surrounding brain parenchyma, leading to later disease recurrence (Wilson et al., 2014). These invasive cells are distinct from their non-invasive counterparts genotypically and phenotypically, and have been found to activate various cellular pathways essential for invasion and migration (Xie, Mittal, & Berens, 2014b). Furthermore, several individual proteins have been implicated in regulating the invasive behaviour of glioma cells. One of these proteins is p75\textsuperscript{NTR}, which our laboratory found to be upregulated in the invasive glioma cells and expressed on a subpopulation of glioma cells in GBM patients’ specimens (A. L. Johnston et al., 2007). Ectopic expression of p75\textsuperscript{NTR} in genetically different non-invasive glioma cells induced their invasive ability in vitro and in vivo (A. L. Johnston et al., 2007). Furthermore, p75\textsuperscript{NTR}-mediated glioma invasion was found to be neurotrophin binding-dependent, and proteolytic processing of p75\textsuperscript{NTR} is essential for this process (A. L. Johnston et al., 2007; L. Wang et al., 2008). Although these observations highlight the cell-autonomous role of p75\textsuperscript{NTR} in glioma invasion, the cell non-autonomous role of p75\textsuperscript{NTR} in glioma invasion specifically and in cancer progression generally is still unknown. Here, we showed that p75\textsuperscript{NTR} is secreted in glioma derived-EVs and induced glioma invasion through paracrine signaling. To assess the cell non-autonomous role of p75\textsuperscript{NTR} in glioma invasion, we used CM to study the secretome of p75\textsuperscript{NTR}-expressing cells. CM of cancer cells contain secreted proteins released through classical and non-classical secretion pathways. CM has limited complexity compared to serum or proximal biological fluids that are in direct contact with the tumor cells,
allowing the identification of low abundance proteins (Pavlou & Diamandis, 2010). In addition, the experimental conditions to prepare CM are highly controlled providing reproducible and quantifiable results. We found that CM of genetically distinct p75<sup>NTR</sup>-expressing human glioma cells is able to confer the invasive ability of non-invasive glioma cells (Figures 4.1B and C), suggesting the presence of a specific molecule(s) that acts as a paracrine mediator of invasion. Our observations that p75<sup>NTR</sup> is detectable in CM of p75<sup>NTR</sup>-expressing cells and the ability of purified EVs, but no other components in CM, to induce glioma invasion suggest that p75<sup>NTR</sup> might package in these EVs and contribute to the glioma invasion through a cell non-autonomous mechanism. The mechanisms that stimulate generation and release of EVs from cancer cells are still not fully understood, but loss of tumor suppressor p53, expression of oncogenic EGFR<sub>vIII</sub>, hypoxic environment and radiation are factors that associated with increased EVs release from cancer cells (Al-Nedawi et al., 2008; Arscott et al., 2013; Kucharzewska et al., 2013; X. Yu et al., 2006). Our results suggest p75<sup>NTR</sup> is also another factor that enhances EV secretion from glioma cells. Although the mechanism by which p75<sup>NTR</sup> regulates EV release is still to be identified, p75<sup>NTR</sup> has been shown to localize within the lipid rafts (Fujitani et al., 2005; C. S. Huang et al., 1999) and this may cause release of p75<sup>NTR</sup> as cargo of raft-related EVs to mediate glioma invasion. The binding of neurotrophins to p75<sup>NTR</sup> activates sphingomyelin hydrolysis with concomitant elevation of ceramide to regulate various biological process including neuron outgrowth, neuronal cell survival and cell death (Brann et al., 1999; Dobrowsky, Werner, Castellino, Chao, & Hannun, 1994). p75<sup>NTR</sup>-dependent ceramide production has been shown in several systems, and as a result, ceramide is often considered a downstream effector of p75<sup>NTR</sup> (Brann et al., 1999; Dobrowsky et al., 1994; Mamidipudi & Wooten, 2002). Interestingly, the production of ceramides from sphingomyelin by sphingomyelinase has been found to trigger the
budding of exosomes, a subpopulation of EVs, into MVBs and release of these vesicles into the extracellular environment (Trajkovic et al., 2008). Collectively, these observations suggest that ceramide signaling downstream of p75NTR enhances EVs release. Consistent with this hypothesis, we found that ectopic expression of p75NTR in glioma cells enhanced their EV release and the formation of vesicular membrane protrusions compared to that in non-p75NTR-expressing cells. Furthermore, we found p75NTR is secreted in EVs released by glioma cells including patient-derived BTICs. These findings are in agreement with other studies showing that p75NTR in sympathetic neurons and PC12 cells is internalized in a clathrin and dynamin-dependent manner, evade the lysosomal route at the level of the early endosome and accumulate in two distinct endosomal populations. These populations are the Rab11-positive recycling endosomes and MVBs that are positive for CD63, a marker of MVBs that fuse with the plasma membrane for exosomal release (Escudero et al., 2014).

In a previous study, we demonstrated that: (1) proteolytic processing of p75NTR occurs in genetically different glioma cell lines, surgically resected tumor specimens, and BTICs isolated from GBM patient specimens; (2) cleavage-resistant alleles of p75NTR are incapable of mediating glioma invasion; and (3) pharmacological inhibition of p75NTR proteolytic processing with a clinically applicable γ-secretase inhibitor results in a dramatic reduction of glioma invasion both in vitro and in vivo and significantly prolonged survival of animals bearing p75NTR-positive intracranial tumors (L. Wang et al., 2008). Here, we found that: (1) p75NTR proteolytic processing is detectable in EVs isolated from genetically different glioma cell lines, patient-derived BTICs and serum of animals bearing p75NTR-positive intracranial tumors; (2) the cleavage-resistant mutant of p75 (p75FasS) is also detectable in EVs isolated from p75FasS-expressing glioma cells (3) inhibition of γ-secretase activity in p75NTR-expressing glioma cells inhibited p75NTR proteolytic
processing and this inhibition was reflected in EVs isolated from these cells; and (4) uptake of p75<sup>NTR</sup>-containing EVs, but not p75FasS-containing EVs or EVs isolated from p75<sup>NTR</sup>-expressing cells treated with γ-secretase inhibitor by non-invasive glioma cells induced their invasive ability.

It is not clear whether p75<sup>NTR</sup> proteolytic processing occurs within EVs independently and whether the γ-secretase complex is also released from cells through EVs. γ-secretase is a membrane multi-subunit protease complex that consists of four essential subunits: presenilin PS (including PS1 and PS2), nicastrin, anterior pharynx defective 1 (APH-1), and presenilin enhancer 2 (Kimberly et al., 2003). Amyloid precursor protein (APP), similar to p75<sup>NTR</sup>, undergoes regulated α-secretase and γ-secretase cleavage to produce amyloid beta-peptide (Aβ), which is strongly implicated in the pathogenesis of Alzheimer's disease (AD) (Wolfe, 2008). EVs isolated from cultured cells expressing wild-type human APP were found to transport both the APP and its cleaved fragment, Aβ (Rajendran et al., 2006; Sharples et al., 2008). These vesicles were also found to transport the α-secretase component (ADAM10) and some, but not all, γ-secretase components including PS1 and PS2 (Sharples et al., 2008). Collectively, these observations with our results suggest that p75<sup>NTR</sup> proteolytic processing occurs within cells, and then the p75<sup>NTR</sup> is transported and released through EVs. In our present study, we showed that p75<sup>NTR</sup> proteolytic processing in EVs is required for inducing glioma invasion by p75<sup>NTR</sup>-containing EVs. Furthermore, we observed that the ICD fragment of p75<sup>NTR</sup> accumulated in these EVs. The exact molecular mechanism(s) by which the ICD fragment of p75<sup>NTR</sup> induces the invasive behavior of glioma cells is still unknown.

As is the case with the Notch signaling pathway (Nam, Weng, Aster, & Blacklow, 2003), several reports suggest that the p75<sup>NTR</sup>-ICD fragment can translocate to the nucleus, but whether it functions as part of a transcriptional complex is still unclear (Frade, 2005; Podlesniy et al., 2006).
In contrast to the observation that suggest p75NTR proteolytic processing is essential for p75NTR-mediated glioma invasion (L. Wang et al., 2008), it has been recently reported that p75NTR mediates glioma migration in vitro in a γ-secretase independent mechanism through regulation of cadherin-11 (Cdh-11), where siRNA-mediated down-regulation of Cdh-11 resulted in a dramatic inhibition of p75NTR-mediated glioma cell migration (J. Berghoff et al., 2015). These findings suggest that p75NTR induces glioma cell migration through both γ-secretase-dependent and independent mechanisms; however, the full complexity of these diverse signaling paradigms is not fully understood.

Importantly, we found proteolytic processed p75NTR within EVs isolated from different patient derived-BTICs and from the serum of animals bearing p75NTR-positive intracranial tumors. This rare population of cells has been shown to have stem-like properties, to be able to repopulate the tumor, and to contribute to the resistance of current GBM therapies (radiation and temozolomide) (Bao et al., 2006; Hemmati et al., 2003; Liu et al., 2006; S. K. Singh et al., 2004); and thus, may represent a “disease reservoir” for these devastating tumors. Unlike other glioma cells, these cells are highly invasive in vivo (Alshehri et al., 2017) and generate tumors that recapitulate the histopathological features of the parental GBM tumors (Bao et al., 2006; Hemmati et al., 2003; Liu et al., 2006; S. K. Singh et al., 2004). EVs derived from glioma cells in general and, BTICs in particular, are likely to represent one of the mechanisms by which these cells modulate the tumor microenvironment and make it more permissive for invasion and growth. Consistent with this hypothesis, it has been recently shown that ovarian cancer cells secrete CD44-enriched EVs that are taken up by human peritoneal mesothelial cells (HPMCs), which are located within the tumor microenvironment, leading to the transfer of CD44 to these cells. The CD44 expression in HPMCs promoted cancer invasion by inducing the HPMCs to secrete matrix
metalloproteinase (MMP9), an enzyme that belongs to the zinc-metalloproteinase family involved in the degradation of the extracellular matrix, and by cleaning the mesothelial barrier for improved cancer cell invasion (K. Nakamura et al., 2017). Another study showed that integrin-αvβ3 is transferred from tumorigenic to non-tumorigenic cancer cells via EVs and its de novo expression in recipient cells promotes cell migration on its ligand (A. Singh et al., 2016). Both CD44 and integrin-αvβ3 are expressed by glioma cells, including BTICs, and contribute to the invasive behaviour of glioma cells (Kwiatkowska & Symons, 2013); therefore, it is possible in the glioma microenvironment that p75NTR, CD44, integrin-αvβ3 and other molecules that induce glioma invasion are transferred between cells through EVs, resulting in the modulation of the tumor microenvironment and making it permissive for glioma cells invasion. Such an exchange process has been studied in vivo using a combination of high-resolution intravital imaging with a Cre recombinase-based method. Zomer et al. (2015) showed that EVs secreted by malignant tumor cells are taken up by less malignant tumor cells located within the local environment and within distant tumors and that these EVs carry mRNAs involved in migration and metastasis. Importantly, intravital imaging showed that the less malignant tumor cells that take up EVs display enhanced migratory behavior and metastatic capacity (Zomer et al., 2015).
Chapter Five: General discussion and conclusions

5.1 Cell autonomous roles of $\text{p75}^{\text{NTR}}$ in glioma progression

GBM tumors, the most common and aggressive primary brain tumor in adults, are believed to be initiated and maintained by BTICs, a subpopulation of cancer cells. BTICS are capable of extensive self-renewal, differentiation towards multiple lineages and recapitulation of the invasive phenotypes of GBM tumors when intracranially transplanted into immunodeficient mice (Galli et al., 2004; Hemmati et al., 2003; S. K. Singh et al., 2003; S. K. Singh et al., 2004). Because BTICs seem to have a fundamental role in initiating GBM tumors and their recurrence, several groups have proposed that definitive GBM treatment needs to target this population (Bao et al., 2006; J. Chen, McKay, et al., 2012). One of the multiple strategies that has been suggested to eradicate cancer stem cells in any cancer is targeting specific surface markers that are expressed by these cells (Dragu, Necula, Bleotu, Diaconu, & Chivu-Economescu, 2015). For instance, CD133 is a largely used marker of cancer stem cells in several cancers; silencing of CD133 in GBM patient-derived BTICs resulted in a dramatic reduction of the self-renewal and tumorigenic capacity of these cells (Brescia et al., 2013). Here, we found $\text{p75}^{\text{NTR}}$ is variably expressed on genetically different GBM patient-derived BTICs and $\text{p75}^{\text{NTR}}$ silencing in two distinct BTIC lines resulted in a substantial reduction in sphere-forming capacity, proliferation, expression of several stem/progenitor cell marker expression in vitro, and inhibition of the tumorigenic potential of these cells in vivo. This inhibition of tumorigenic potential of $\text{p75}^{\text{NTR}}$-knockdowned BTICs in vivo resulted at least in apart from a significant reduction of the percentage of proliferative cells, increased cell death, and differentiation toward the astrocytic lineage of these cells. As a result, the median survival time of animals bearing brain tumors generated by $\text{p75}^{\text{NTR}}$ knockdown BTICs was significantly extended compared to that of control animals. Moreover, ectopic expression of
p75NTR in genetically different BTIC lines enhanced their sphere-forming capacity, proliferation and increased expression of the stem cell markers CD133 and CD15 in vitro. Moreover, it increased the cells’ invasion both in vitro and in vivo. All together, these findings suggest that p75NTR is a key regulator in maintaining stemness status of BTICs.

The concept of p75NTR playing a role in stem cell biology is not unprecedented. p75NTR is expressed in stem cells from a wide range of different tissues and has been largely utilized to isolate stem/progenitor cells from both normal and pathological tissues (Tomellini et al., 2014). In the early development of mouse embryos, p75NTR was expressed on cells at the early blastocyst stage as well as in mouse pluripotent embryonic germ stem cells in vitro. and mediated the cells’ proliferation (Moscatelli, Pierantozzi, Camaioni, Siracusa, & Campagnolo, 2009). This suggested an important role of p75NTR in early embryo development as it is expressed on stem cells that act as foundations of stem cell hierarchy. p75NTR was also used as a robust marker to isolate neural crest stem cells, a transient population of multipotent stem cells that migrate extensively into the embryo to eventually give rise to a wide range of cell types including glia and neurons of the peripheral nervous system, skeletal cells, cartilage, and adrenal and thyroid glands among others (Betters et al., 2010). In adults, p75NTR was also expressed by distinct stem/progenitor cells including liver stem cells (stellate cells), muscle stem cells (satellite cells), bone marrow stem cells, and oral and esophageal mucosa stem cells (reviewed in (Tomellini et al., 2014). Like their stem cell counterparts in normal tissue, tumor-initiating cells in different cancers have been reported to express p75NTR, and its expression has been implicated in maintenance and potential tumorigenicity of these cells. Similar to what we showed here about the role of p75NTR in GBM patient-derived BTICs, p75NTR was used as a potential marker to isolate melanoma initiating cells that were able to maintain their self-renewal and sustain long-term tumor growth in vivo; the strong
expression of p75NTR in melanoma patient biopsies was associated with poor prognosis for melanoma patients. However, recent studies have shown that p75NTR is not a selective marker for melanoma initiating cells and suggested that this cell surface marker is associated with proliferative and invasive cell populations in melanoma (A. D. Boiko et al., 2010; Boyle et al., 2016; Cheli et al., 2014; Civenni et al., 2011). Moreover, esophageal squamous cell carcinoma p75NTR-expressing cells were found to have a high capacity of self-renewal and were involved in the chemotherapy resistance (S. D. Huang et al., 2009). Collectively, these results suggest that p75NTR could work as a “fate decision” protein that helps maintain potency of stem cells, and when required, enables the cell differentiation program. These dual functions are cell-type specific and must be independently determined for each cellular context.

5.1.1 Migration and invasion or proliferation dichotomy (Go or Grow mechanism): p75NTR as a regulator
The quick progression of GBM tumors largely results from two main hallmarks of cancer: infiltrative growth and enabling replicative immortality. Although extensive cell migration and uncontrolled cell proliferation are two of the major characteristics of GBM progression, it is not clear if migration and proliferation are mutually exclusive phenotypes at the single level or there is phenotype-switching taking place in these cells (Xie et al., 2014b). In vivo imaging of glioma cells showed that they migrate in a saltatory fashion. When migratory glioma cells need to proliferate, they pause for as short as an hour to divide before the daughter cells resumed migration again. The vast majority of glioma cell divisions occurred at or near vascular branch points, indicating that mitosis is regulated by local environmental cues (Farin et al., 2006). Furthermore, close examination of selected GBM cell sub-clones that are highly proliferative or highly invasive or displayed both invasive and proliferative phenotypes, revealed that while the proliferative cells
were able to display anchorage-independent growth in soft agar and were highly tumorigenic when xenografted in immunocompromised mice, the highly invasive cells were exclusively activated for invasion, migration and branching morphogenesis. In response to hepatocyte growth factor/scatter factor (HGF/SF), the highly proliferative cells signaled through Myc, the invasive cells signaled through the MAPK pathway, and the sub-cloned cells displaying both invasive and proliferative phenotypes signaled through both Myc and MAPK pathways, suggesting that both pathways can cooperate to confer both invasive and proliferative phenotypes on tumor cells (Gao et al., 2005).

In this study, we found that down-regulation of p75\textsuperscript{NTR} in genetically diverse BTICs inhibited their proliferation both \textit{in vitro} and \textit{in vivo} but to a lesser extent affected their invasion. On the other hand, in this and previous studies, ectopic expression of p75\textsuperscript{NTR} in genetically different glioma cells including BTICs increased their invasion but to a lesser extent their proliferation (Ahn et al., 2016; A. L. Johnston et al., 2007; L. Wang et al., 2008). These diverse functions of p75\textsuperscript{NTR} may be a result of the presence of different p75\textsuperscript{NTR} co-receptors and neurotrophin ligands, changes in p75\textsuperscript{NTR} intracellular interactors, and changes in the balance of cues from various regulating factors in the local environment.

\textbf{5.1.1.1 p75\textsuperscript{NTR} co-receptors}

In order to understand the molecular mechanisms by which p75\textsuperscript{NTR} mediates proliferation or induces enhancement of glioma migration and invasion, the involvement of a co-receptor must be determined. The Trk receptors (TrkA, TrkB and TrkC), sortilin, the Nogo receptor (NgR) and LINGO-1 are the co-receptors presently known to signal in association with p75\textsuperscript{NTR}. The neurotrophin family are the ligands that can bind to, and transduce signals through the p75\textsuperscript{NTR} and Trk receptors. p75\textsuperscript{NTR} modulates the specificity and affinity of binding between different neurotrophins and Trk receptors (Hantzopoulos et al., 1994; Bibel et al., 1999; Vesa et al.,
2000b; Mischel et al., 2001). Although further experimental validations need to be done, microarray analysis of tumor cells isolated from brain tumors generated by implanting BT147p75\textsuperscript{NTR} KD cells or their BT147 control counterpart into the brains of SCID mice showed that BT147p75\textsuperscript{NTR} KD cells upregulated expression of TrkB and the neurotrophin BDNF compared to the BT147 control cells. These results suggest that these cells escaped cell death mediated by p75\textsuperscript{NTR} knockdown through upregulation of TrkB and its ligand BDNF that may act in autocrine signaling to mediate cell survival of these cells. In agreement with this hypothesis, it has been shown that BTICs express TrkB, TrkC and their ligands NGF, BDNF and NT3. Specific activation of of TrkB and TrkC receptors by ligands BDNF and NT3 on these cells enhanced their cell viability through activation of ERK and Akt pathways. In contrast, knockdown of TrkB and TrkC or pharmacological inhibition of Trk signaling attenuated neurotrophin-dependent ERK activation and inhibited BTIC growth (Lawn et al., 2015).

The dual role of p75\textsuperscript{NTR} in regulating proliferation and invasion of cancer cells is not limited to glioma cells but extends to other cancers. Recently, it has been shown that p75\textsuperscript{NTR} is a key regulator of the dynamic switch of melanoma cells from a high-proliferative/low-invasive state to a low-proliferative/high-invasive state (Restivo et al., 2018). The authors showed that the dynamic modification of p75\textsuperscript{NTR} expression allows melanoma cells to proliferate and divide when the level of p75\textsuperscript{NTR} is low. When reduced growth and increased migration and invasion of these tumor cells are required, the cells express high levels of p75\textsuperscript{NTR}. When these tumor cells metastasized to a distant site, they reduce their invasion and migration signaling and trigger proliferation signaling. The p75\textsuperscript{NTR}-cleaved intracellular domain (ICD) reduced proliferation while interaction of p75\textsuperscript{NTR} and TrkA mediated adhesion via dynamic regulation of a group of cholesterol biosynthesis genes that are required for patient survival (Restivo et al., 2018). Although
these results suggest that p75NTR is a key regulator of phenotype switching in melanoma cells, it is still unknown if p75NTR can regulate the dynamic switch between proliferative and invasive phenotypes on one population of glioma cells or if it regulates both phenotypes independently on different glioma cell populations.

The role of p75NTR in cellular proliferation has also been reported in other cancers. Overexpression of p75NTR or treating p75NTR-expressing cells with BDNF and NT4/5 increases the proliferation and cell survival of breast cancer cells (Descamps et al., 2001; Verbeke et al., 2010). p75NTR also regulates proliferation and migration of hypopharyngeal cancer cells and the knockdown of p75NTR in these cells completely suppressed their ability to form tumors in vivo (Mochizuki et al., 2016). Moreover, p75NTR was reported as a functional marker of tumor-initiating cells in head and neck squamous cell carcinoma and loss of function of p75NTR in these cells inhibited their capacity to form tumors in vivo (Murillo-Sauca et al., 2014). Collectively, these results suggest that p75NTR is a key regulator of cell proliferation and migration and acts as a marker for cancer stem cells in different cancers including glioma, melanoma, breast cancer, and head and neck squamous cell carcinoma.

In a previous study, we showed that neurotrophin-dependent regulated intramembrane proteolysis (RIP) of p75NTR is required for p75NTR-mediated glioma invasion and inhibition of this cleavage process with clinically applicable γ-secretase inhibitors resulted in a dramatic reduction of glioma cell invasion in vitro and in vivo (L. Wang et al., 2008). Interestingly, neurotrophin-RIP of p75NTR was also found to potentiate Trk signaling (Ceni et al., 2010). The authors showed that RIP of p75NTR occurs by means of Trk-dependent activation of MEK-ERK signaling and induction of α-secretase activity, and is independent of neurotrophins binding to p75NTR. Importantly, PC12 cells and neuron-lacking p75NTR showed defects in neurotrophin-dependent activation of Akt
signaling, a phenotype that was rescued by the ectopic expression of full-length p75\textsuperscript{NTR} or the p75\textsuperscript{NTR} intracellular domain (Ceni et al., 2010). Similarly, treatment of BTICs expressing p75\textsuperscript{NTR} and TrK receptors (TrkA, TrkB and TrkC) with exogenous neurotrophins induced cleavage of p75\textsuperscript{NTR} and induced proliferation of these cells, phenotypes that were blocked by the inhibition of Trk signaling (Forsyth et al., 2014). Our results showed important roles of p75\textsuperscript{NTR} in sphere-forming capacity, proliferation, differentiation and tumorigenic behaviour of patient-derived BTICs; however, it is important in the future to investigate if there is any role of Trk receptors in inducing these phenotypes.

5.1.1.2 p75\textsuperscript{NTR} intracellular interactors

p75\textsuperscript{NTR}, similar to other members in the tumor necrosis factor receptor (TNFR) family, lacks intrinsic enzymatic activity and its signaling is mediated by the recruitment of intracellular binding proteins, resulting in the activation of distinct signaling pathways that regulate cell survival, apoptosis, cell cycle arrest and neurogenesis (Tomellini et al., 2014). The family of TNFR-associated factors (TRAFs) is one group of p75\textsuperscript{NTR} interactor proteins that mediate activation of the stress kinase c-Jun N-terminal kinase (JNK) and of the transcription factor NF-κB (Reviewed in (Kraemer, Yoon, & Carter, 2014). TRAFs 1–6 interact with p75\textsuperscript{NTR} and in particular, TRAF2, 4 and 6 regulate p75\textsuperscript{NTR}-induced cell death through interactions with the intracellular domain of p75\textsuperscript{NTR} (Khursigara, Orlinick, & Chao, 1999; Ye et al., 1999). Another intracellular binding partner of p75\textsuperscript{NTR} that also activates JNK signaling is the neurotrophin receptor-interacting MAGE homolog, NRAGE (also known as dlxin or Maged1) (Salehi, Xanthoudakis, & Barker, 2002). The function of NRAGE is not well-understood, but it does appear to contribute to cell cycle regulation and modulation of apoptosis (Salehi et al., 2002). p75\textsuperscript{NTR}-associated cell death executor (NADE) is another intracellular protein that interacts with the
intracellular domain of p75NTR and its overexpression together with p75NTR in HEK 293 cells induces apoptosis (Mukai et al., 2000). Although most studies showed a role for p75NTR in regulating programmed cell death was primarily observed in neuronal cell types, it is now known that p75NTR can induce cell death of both neuronal and non-neuronal cell types and is involved in the pathogenesis of several neurodegenerative diseases including Alzheimer’s disease (AD), amyotrophic lateral sclerosis and Parkinson’s disease (Reviewed in (Kraemer et al., 2014). In terms of cancer, although p75NTR promotes cell survival and tumor growth in several cancers including glioma (Forsyth et al., 2014), melanoma (A. D. Boiko et al., 2010), breast cancer (Kim et al., 2012), head and neck squamous cell carcinoma (S. D. Huang et al., 2009) and hypopharyngeal carcinoma (Mochizuki et al., 2016), it has been reported that p75NTR can act as a tumor suppressor by inhibiting proliferation, blocking cell cycle progression and inducing cell death of gastric and prostatic cancer cells (Jin et al., 2007; Krygier & Djakiew, 2002). However, as in the case of glioma and in agreement with what we showed in this study, p75NTR tends to promote cell survival and tumor growth in the majority of tumors investigated thus far. The ability of p75NTR to promote or suppress tumor growth highlights the differences in neurotrophin signaling based on the cellular context in which it is deciphered.

Herein, we showed that knockdown of p75NTR in genetically different BTICs reduced their proliferation, induced cell cycle arrest and increased sensitivity to apoptotic cell death. Although we currently do not know the signaling downstream of p75NTR that is involved in inducing cell cycle arrest and apoptosis in p75NTR knockdown BTICs, microarray array data of these cells revealed a global down-regulation in gene expression of many genes involved in controlling cell cycle progression such as cyclin A, cyclin D1, cyclin E and PCNA. Interestingly, these genes were downregulated upon cell cycle arrest caused by ectopic expression of p75NTR in human gastric
cancer cells (Jin et al., 2007). These results suggest that the downregulation of genes that regulate cell cycle progression may be involved in cell cycle arrest and apoptotic cell death induced by p75NTR knockdown in BTICs.

In agreement with our previous findings about the role of p75NTR in mediating glioma invasion (Ahn et al., 2016; A. L. Johnston et al., 2007; L. Wang et al., 2008), ectopic expression of p75NTR in genetically distinct patient-derived BTICs induced their invasion in vitro and in vivo. Intracellular interactor proteins of p75NTR can also regulate p75NTR-induced invasion and migration. Using a peptide-based affinity strategy utilizing the PDZ-binding motif of p75NTR, PDLIM1, a protein involved in cytoskeleton regulation, was identified as a novel signaling adaptor for p75NTR (Ahn et al., 2016). Our laboratory showed that PDLIM1 interacts with p75NTR in highly invasive p75NTR-expressing human glioma cells and patient-derived BTICs, and conversely, downregulation of PDLIM1 or the complete loss of the PDZ-binding motif (ΔSPV) resulted in inhibition of invasion (Ahn et al., 2016). Moreover, the interaction between PDLIM1 and p75NTR was found to be highly regulated as prevention of phosphorylation on serine 303 located in the cytoplasmic tail of p75NTR by pharmacological inhibition of the cAMP-dependent protein kinase (PKA), or by a mutational strategy (S303G), abrogated p75NTR-mediated glioma invasion and resulted in phosphorylation of serine 425 within the C-terminal PDZ-binding motif (SPV) (Ahn et al., 2016). This finding confirms the importance of the interaction between p75NTR and PDLIM1 in regulating glioma cell invasion. In conclusion, all discussed findings in this section showed the essential role of p75NTR interactor proteins in controlling cell survival, cell death and invasion of cells in p75NTR-dependent manners.
5.1.1.3 Microenvironment regulation: crosstalk between tumor and host

In order to elucidate the mechanism by which downregulation of p75\textsuperscript{NTR} in BTICs inhibited tumor growth and induced cell cycle arrest and apoptotic cell death, and conversely, how ectopic expression of p75\textsuperscript{NTR} in BTICs induced tumor cell invasion, the role of the tumor microenvironment in regulating these phenotypes must be determined. In the GBM tumor microenvironment, the dynamic crosstalk between tumor cells and the normal host tissues regulates the progress of tumor cell invasion and proliferation. Recently, a group of tumor extracellular matrix (ECM) factors, chondroitin sulfate proteoglycans, were shown to play a major role in regulating phenotype switching of GBM cells from migration and invasion (“go”) to proliferation (“grow”) (Silver et al., 2013). Consistent with these findings, we recently showed that the highly invasive p75\textsuperscript{NTR} glioma cells were associated with striking changes in the tumor microenvironment generally and marked changes in the composition and rigidity of the tumor associated-ECM particularly (Alshehri et al., 2017). These changes include the presence of more collagen within the highly invasive p75\textsuperscript{NTR} glioma tumor microenvironment as detected by second-harmonic imaging and immunohistochemical analysis of collagen using Picrosirius Red staining (Alshehri et al., 2017). Interestingly, microarray analysis of BT147 control and BT147p75\textsuperscript{NTR} showed that p75\textsuperscript{NTR} downregulation was associated with changes in gene expression patterns of several genes associated with the composition of tumor-associated ECM including downregulation of gene expression of collagen IV (COL4A1 and COL4A2), collagen 9 (COL9A3) and laminin (LAMB1 and LAMC1). Expression of collagen and laminin have been implicated in glioma invasion and progression (Payne & Huang, 2013). Importantly, laminin has been used to expand BTICs in adherent cultures while maintaining their stem cell properties (Pollard et al., 2009). Collectively, these findings suggest that expression of p75\textsuperscript{NTR} on glioma cells is associated with changes in the
tumor ECM components that may trigger the phenotypic switching from “go” to “grow” in a 
p75\textsuperscript{NTR}-dependent manner.

There has also been considerable interest surrounding the concept that the slowly dividing 
invasive glioma disease reservoir and the proliferating glioma disease reservoir may be very 
distinct metabolic states. Proliferating tumors, such as GBM, use anaerobic glycolysis regardless 
of the amount of oxygen availability, a phenomenon known as the Warburg effect, which 
consumes less oxygen but produces large amounts of lactate and alanine (Hanahan & Weinberg, 
2011). Anaerobic glycolysis plays a dual role in regulating both tumor invasion and proliferation 
(Gatenby & Gillies, 2004). An acidic GBM tumor microenvironment results from the excessive 
lactate produced by fast-growing glioma cells contributes to the death of normal neuronal and glial 
cell populations and facilitates degradation of the ECM by matrix metalloproteinases produced by 
glioma cells to induce their migration (Gatenby & Gillies, 2004). Whether p75\textsuperscript{NTR} or neurotrophin 
signaling in general is involved in regulating metabolism in glioma is still unclear; however, 
microarray analysis of BT147 control and BT147p75\textsuperscript{NTR} KD cells showed striking changes in gene 
expression of glucose and glutamate transporters in p75\textsuperscript{NTR} knockdown cells including 
upregulation of gene expression of glucose transport 5 (GLUT5) and downregulation of 
hexokinase 1 (HK1) and glutaminase (GLS). These findings suggest a novel role of p75\textsuperscript{NTR} in 
regulating metabolic activities of glioma cells. Interestingly, our laboratory found recently that 
p75\textsuperscript{NTR} regulates metabolic state of glioma cells including BTICs and targeting p75\textsuperscript{NTR} expression 
in these cells reprogrammed their metabolic state, shifting from a dependence on glutamine to one 
that requires glucose (Ahn et al; unpublished paper). Moreover, p75\textsuperscript{NTR} has been found to regulate 
energy expenditure in the setting of obesity via the regulation of cAMP levels by PKA (Baeza- 
Raja et al., 2016), similar to what we have seen with p75\textsuperscript{NTR} and PDLIM1 (Ahn et al., 2016).
Our observations that many apoptotic tumor cells present in the brains of mice bearing tumors generated by either BT147p75<sup>NTR</sup> KD or BT48p75<sup>NTR</sup> KD cells but not their controls BT147 and BT48 control cells, raised the question about the presence of specific stresses in these tumor microenvironments. One possibility for the dramatic increase in the number of apoptotic tumor cells in these microenvironments is the presence of large amounts of death ligands such as TNF-α, FasL and TNF-related apoptosis-inducing ligand (TRAIL). For instance, it has been shown that TRAIL induced cell death of glioma cells by activation of caspase-8 and -3, poly (ADP-ribose) polymerase cleavage, and DNA fragmentation (Hao et al., 2001). Furthermore, TRAIL-induced apoptosis in glioma cells was enhanced by Akt inhibition (Puduvalli et al., 2005). Interestingly, pathway analysis acquired from microarray data of tumor BT147p75<sup>NTR</sup> KD cells isolated from brains of mice bearing brain tumors generated by these cells showed a dramatic inhibition of Akt signaling pathway in these cells. Whether these death ligands are elevated in the tumor microenvironment of p75<sup>NTR</sup> knockdown cells requires further investigation.

In conclusion, the findings of this study suggest that p75<sup>NTR</sup> regulates both proliferation and invasion of glioma cells including patient-derived BTICs by interacting with p75<sup>NTR</sup> co-receptors and distinct intracellular adaptor proteins and regulating the tumor microenvironment. Therefore, understanding p75<sup>NTR</sup> signaling in regulating GBM proliferation and invasion may open avenues to discoveries that may help in the differential diagnosis and novel treatment of this devastating disease.

5.1.2 Limitations and future directions

In this study, sphere-forming assays were used to retrospectively to identify stem cells based on their capacity to maintain self-renewal at the single cell level in vitro. Although sphere-forming assays are widely used in stem cell biology, they do have limitations and the results of the
assays should be interpreted with caution. Seeding cell density is one of the most crucial of sphere-forming assays because it has important impacts on cellular clonality, as each sphere is considered to be derived from a single cell and is therefore a single clone (Pastrana, Silva-Vargas, & Doetsch, 2011). The final readout of sphere-forming assays is the number and size of spheres, and results obtained from high density seeded cultures are impossible to interpret because of fusion of spheres, leading to misinterpretations. In this study, we optimized the seeding cell density and used a 2000 cell/well in 96-well plate to avoid cell fusion and aggregation. Another caveat of sphere forming assays is that they may not detect quiescent and slow dividing stem cells. On the other hand, these assays allow quick expansion of cells that are actively dividing. Furthermore, both stem cells and their progenies have the capacity to give rise to neurospheres. Therefore, sphere forming assays may not be the ideal assays to reflect the frequency of stem cells in vivo, but they reflect the ability of specific cell populations to exhibit stem cell characteristics. Less than 6% of cells in neurospheres can be passaged more than seven times, suggesting that cells that exhibit extensive self-renewal only represent a small fraction of the whole cell population (S. A. Louis et al., 2008).

In this study, we also assessed the effects of p75NTR loss of function and p75NTR gain of function on the expression levels of stem/progenitor cell markers both in vitro and in vivo. One of the caveats of assessing these markers and associating their expression with cells exhibiting stem cell traits is that these markers are dynamic, and their expressions are regulated by growth conditions. For instance, self-renewing CD133- glioma cells are able to generate both CD133+ and CD133- glioma cells (R. Chen et al., 2010). In addition, hypoxic conditions have been implicated in the dynamic nature of these receptors leading to an increase in the proportion of glioma stem cells expressing CD133 and a decrease in the proportion of CD44 expressing cells (Brown et al., 2017). In this study, we used different markers both in vitro and in vivo and
confirmed our results in genetically different BTICs to confirm that observed phenotypes were the result of \(p75^{\text{NTR}}\) loss or gain of function in these cells.

Neural stem cells (NSCs) were shown to propagate as neurospheres \textit{in vitro} in the presence of high levels of growth factors, specifically EGF at 20 ng/ml, and bFGF at 10ng/ml (Reynolds & Weiss, 1992). These culture conditions were used in this study to propagate BTICs as neurospheres. Although using high levels of EGF in the medium of these culture conditions is still the gold standard method for \textit{in vitro} culture of NSCs and BTICs, such high concentrations of EGF may heavily bias cultured cells towards glial differentiation, both \textit{in vitro} and after transplantation \textit{in vivo}, but on the other hand, lowering the EGF concentration is associated with more neuronal differentiation (Burrows, Wancio, Levitt, & Lillien, 1997). To avoid such caveat, in this study we always passaged BTICs in freshly prepared homemade NSC medium including freshly prepared EGF and bFGF.

Another limitation of this study is that the microarray analysis was performed on tumor cells isolated from mice bearing brain tumors generated by BT147p75\textsuperscript{NTR} KD cells or their controls BT147 cells only. The reason for not doing microarray on tumor cells isolated from mice bearing brain tumors formed by BT48p75\textsuperscript{NTR} KD and their control BT48 cells is that the animals bearing tumor formed by BT48p75\textsuperscript{NTR} KD have a very long survival time (1.4 year) and still have not developed any sickness signs till the time of writing this thesis.

Although the current study identified novel roles of \(p75^{\text{NTR}}\) in regulating sphere-forming capacity, proliferation, cell cycle progression, differentiation and invasion of genetically distinct patient-derived BTICs, future work is required to determine if different \(p75^{\text{NTR}}\) ligands, coreceptors, intracellular interactors and microenvironmental regulators are also involved in regulating these phenotypes. Furthermore, future research needs to focus on validating microarray
data that indicate a possible role of p75\textsuperscript{NTR} in regulating GBM metabolism, angiogenesis and modulation of ECM components and rigidity.

**5.2 Cell non-autonomous roles of p75\textsuperscript{NTR} in glioma invasion**

GBM is the most common, complex, treatment resistant and deadliest type of brain cancers in adults. Despite the advances that have been made in surgical, pharmacological, and radiation therapeutic approaches, GBM is still incurable because of its diffuse infiltration into adjacent healthy brain tissue. The invasive behavior of glioma cells has been associated with the expression of several proteins. One of these proteins is p75\textsuperscript{NTR} that our laboratory found to be upregulated in invasive glioma cells and expressed on a subpopulation of glioma cells in GBM patients’ specimens (A. L. Johnston et al., 2007). p75\textsuperscript{NTR} ectopic expression in genetically distinct non-invasive glioma cells induced their invasive ability \textit{in vitro} and \textit{in vivo} (A. L. Johnston et al., 2007). Furthermore, p75\textsuperscript{NTR}-mediated glioma invasion was found to be neurotrophin-binding dependent and proteolytic processing of p75\textsuperscript{NTR} was shown to be essential for this process (A. L. Johnston et al., 2007; L. Wang et al., 2008). Although these findings addressed the cell autonomous role of p75\textsuperscript{NTR} in glioma invasion, the cell non-autonomous role of p75\textsuperscript{NTR} in glioma invasion specifically and in cancer progression generally is still unknown. Herein, we found that p75\textsuperscript{NTR} is transferred between glioma cells via small glioma cell-derived vesicles known as EVs. The uptake of p75\textsuperscript{NTR}-containing EVs by non-invasive glioma cells induced their invasive behaviour to levels similar to parental p75\textsuperscript{NTR}-expressing cells. We found that the RIP of p75\textsuperscript{NTR} is detectable in glioma cell derived EVs and this process is required for mediating glioma invasion by p75\textsuperscript{NTR}-containing EVs, because treating non-invasive glioma cells with EVs isolated from cleavage-resistant chimeras of p75\textsuperscript{NTR} or from p75\textsuperscript{NTR}-expressing cells treated with a \(\gamma\)-secretase inhibitor did not have a
significant impact on glioma invasion. Moreover, p75<sup>NTR</sup> was detected in EVs isolated from serum of mice bearing p75<sup>NTR</sup>-positive intracranial tumors.

The p75<sup>NTR</sup> signaling cascade is a complex signaling axis that results in activating various biological functions based on different factors, including cell type, cellular context, neurotrophin binding, the presence of p75<sup>NTR</sup> co-receptors, intracellular protein interactors of p75<sup>NTR</sup>, post-translational modification and microenvironmental regulators (Schor, 2005). The different cellular responses that are activated by p75<sup>NTR</sup> signaling include cell survival, cell death, cell cycle regulation, apoptosis, neurite outgrowth and retraction, myelination, cell migration and invasion and progenitor differentiation (Reviewed in (Kraemer et al., 2014). Although these diverse cellular activities highlight the importance of cell autonomous roles of p75<sup>NTR</sup> in different cellular systems, cell non-autonomous roles of p75<sup>NTR</sup> in developmental and pathological conditions are still largely unknown. Our observations that p75<sup>NTR</sup> is transferred between glioma cells through EVs revealed a novel cell non-autonomous mechanism of p75<sup>NTR</sup> by which p75<sup>NTR</sup> induced glioma invasion. We found that p75<sup>NTR</sup> expression in glioma cells was associated with a dramatic increase of EV release from these cells. Although the molecular mechanism that triggers increased release of EVs in p75<sup>NTR</sup>-dependent manner is still unknown, neurotrophin-binding to p75<sup>NTR</sup> triggers sphingomyelin hydrolysis leading to increased production of ceramides, lipid molecules that play a major role in the biogenesis and release of EVs (Brann et al., 1999; Trajkovic et al., 2008).

p75<sup>NTR</sup>, similar to several other proteins including Notch, the amyloid precursor protein, and ErbB4 receptor tyrosine kinase, undergoes regulated α-secretase and γ-secretase cleavage, a mechanism known as RIP (K. C. Kanning et al., 2003; Pardossi-Piquard et al., 2005; Podlesniy et al., 2006). Initially, p75<sup>NTR</sup> is cleaved by a wide range of metalloproteinases such as α-secretase to shed the extracellular domain (ECD) leaving an unstable membrane bound C-terminal fragment.
(CTF) that is further cleaved by a presenilin-dependent γ-secretase to release an Intracellular domain (ICD) with potential signaling capacity (DiStefano et al., 1993; Kevin C Kanning et al., 2003). As mentioned previously, our laboratory showed that neurotrophin-dependent RIP of p75NTR is required for the cell autonomous mechanism of p75NTR in mediating glioma cell invasion and this proteolytic processing of p75NTR was detected in several p75NTR-positive patient tumor specimens and BTICs (L. Wang et al., 2008). Interestingly, blocking RIP of p75NTR genetically by expressing cleavage resistant chimeras of p75NTR or pharmacologically using clinically relevant γ-secretase inhibitors dramatically reduced the highly invasive nature of genetically distinct glioma cells and patient-derived BTICs in vitro and in vivo (L. Wang et al., 2008). Here, we found that proteolytic processing of p75NTR is detectable in EVs isolated from glioma cells including patient-derived BTICs. Importantly, proteolytic processing of p75NTR in EVs was required to induce invasion of p75NTR-containing EV recipient cells. This proteolytic processing of p75NTR was inhibited in both p75NTR glioma cells and their derived EVs treated with a clinically relevant γ-secretase inhibitor. These findings suggest that targeting γ-secretase will not only inhibit glioma cell invasion mediated by a cell autonomous mechanism of p75NTR, as we showed previously (L. Wang et al., 2008), but also can inhibit glioma cell invasion induced by a cell non-autonomous mechanism of p75NTR. Since γ-secretase plays a major role in the abnormal accumulation of amyloid β-peptides, which leads to the formation of plaques that are believed to be the pathogenesis of Alzheimer disease (AD) (D’Onofrio et al., 2012). Given the connection between AD and γ-secretase, γ-secretase inhibitors were used in two large Phase III clinical trials in mild-to-moderate AD patients but these trials were prematurely interrupted because of detrimental cognitive and functional effects of the drugs, possibly due to the inhibition of the cleavage of Notch, a transmembrane receptor that plays a major role in regulating cell-fate decisions.
Currently, new γ-secretase inhibitors are being developed with the hope of overcoming the previous setbacks (D'Onofrio et al., 2012). In contrast to our observations that suggest p75NTR proteolytic processing is essential for inducing glioma cell invasion by both p75NTR cell autonomous and cell non-autonomous mechanisms, it has been recently shown that p75NTR mediates glioma migration in vitro in a γ-secretase independent mechanism through regulation of cadherin-11 (Cdh-11) (J. Berghoff et al., 2015). The downregulation of Cdh-11 resulted in a marked inhibition of p75NTR-mediated glioma cell migration. Collectively, all the discussed findings suggest that p75NTR mediates glioma cell migration and invasion through both γ-secretase-dependent and independent mechanisms; however, the full complexity of these diverse signaling paradigms has not been fully identified.

EVs have been implicated in the progression of GBM tumors through cell-to-cell transfer of multiple proteins, specialized lipids, and selected nucleic acids. For instance, cells expressing the mutated EGFR variant III (EGFRvIII), a gain-of-function mutation that arises from genomic deletion of exons 2–7 resulting in a ligand-independent constitutively active receptor, released EVs containing this receptor, which were then incorporated into cells that lack this onco-protein, resulting in inducing oncogenic activity in these cells through the activation of transforming pathways such as Akt and Mitogen-activated protein kinase (MAPK) (Al-Nedawi et al., 2008). Similarly, in this study we found p75NTR transferred between glioma cells, including BTICs, through EVs, and the uptake of p75NTR-containing EVs by non-invasive glioma cells induced their invasive behaviors dramatically. The role of glioma cell-derived EVs in mediating glioma cell invasion has been highlighted in several studies. For instance, the p75NTR co-receptor TrkB can be transferred between glioma cells through EVs to restore proliferation and invasion capacity of glioma cells expressing low levels of YKL-40, glycoprotein that is involved in regulating
proliferation and invasion of glioma cells (Pinet et al., 2016). Furthermore, glioma cell-derived EVs contribute to glioma cell migration and invasion by transferring matrix metalloproteinases (MMPs) between glioma cells. MMPs contribute to glioma cell invasion through the degradation of ECM in the brain, making the brain microenvironment more permissive for glioma cells to infiltrate and migrate through brain parenchyma (Nakada et al., 2003). Interestingly, glioma cells grown under hypoxic conditions release EVs enriched with MMPs, particularly MMP9, and the uptake of these EVs mediated secretion of several potent growth factors and cytokines and stimulated migration of recipient cells (Kucharzewska et al., 2013). Collectively, these observations suggest that glioma cell-derived EVs contribute to glioma progression through transfer of different proteins, specialized lipids and selected nucleic acids.

EVs may represent valuable diagnostic and prognostic markers for the progression of GBM tumors and other cancers. The presence of EVs in the sera of GBM patients carrying the mutant EGFRvIII mRNA in tumors opened the door for using EV contents in patients’ biofluids as biomarkers for brain tumors and other cancers (Skog et al., 2008). Other potential biomarkers that can provide information on GBM progression and response to therapy were also detected in EVs isolated from sera or cerebrospinal fluid of GBM patients. These potential markers include mutations in isocitrate dehydrogenase (IDH) mRNA (W. W. Chen et al., 2013), elevated miR-21 levels (Akers, Ramakrishnan, et al., 2013) and changes in the transcriptome profile of EV mRNA(L. Muller et al., 2015). In this study, we detected p75NTR in EVs isolated from sera of mice bearing brain tumors formed by patient-derived BTICs expressing p75NTR. Although p75NTR-containing EVs may not serve as diagnostic or prognostic markers of GBM tumor due to lack of specificity, it is confirmed that p75NTR is transferred by EVs in vivo. Whether these p75NTR-containing EVs have impact on tumor growth and glioma cell invasion in vivo is still under
investigation. All together, these findings suggest that glioma cell-derived EVs may serve as GBM diagnostic and prognostic biomarkers. However, deep-sequencing and high-resolution technologies have to be developed to accurately detect potential markers in EVs of GBM patients.

### 5.2.1 Limitations and future directions

Although this study identified a novel role of p75\textsuperscript{NTR} in mediating glioma invasion through its transfer between glioma cells through EVs, there are several limitations of this study and more research needs to be conducted to understand the molecular mechanisms by which EVs induce glioma invasion.

EVs are heterogeneous populations that are produced by almost all cells, have different means of biogenesis, carry diverse cargoes, and change dynamically in number and content in response to environmental and physiological conditions (Yanez-Mo et al., 2015). The classification of EV subtypes is still ongoing and thus far includes exosomes (30 to 100 nm in diameter), which are released from cells upon fusion of an intermediate endocytic compartment, the multivesicular body (MVB), with the plasma membrane; microvesicles (100 nm to 1 µm in diameter), which bud from the cell surface, and apoptotic blebs (ranging from 1 µm to >2 µm in diameter), which are generated by dying cells (Yanez-Mo et al., 2015). One of the main questions remaining in the EV field and in almost every study about the role of EVs physiologically or pathologically is: which EVs subtype is responsible for mediating physiologically or pathologically the phenotypes in EVs recipient cells? The complexity of isolating a pure specific population of EVs arises from considerable overlap of physicochemical and biochemical properties of different EV populations, the heterogeneity that exists between the single EV subtypes and the lack of reliable isolation methods that selectively yield the isolation of a specific EV subtype (P. Li, Kaslan, Lee, Yao, & Gao, 2017). Specific characterizations have been proposed...
for each EVs subtype, but currently there is still a lack of unique markers that can distinguish specifically these EV subtypes (Yanez-Mo et al., 2015). Because no clear criteria currently exist to distinguish, isolate, and identify different EV populations, the term extracellular vesicles (EVs) was introduced by International Society of Extracellular Vesicles (ISEV). To improve the credibility, reliability, reproducibility of the reported findings, ISEV has recommended minimal requirements for EVs definition including the minimal experimental requirements for definition of EVs and their function criteria (Lotvall et al., 2014). Furthermore, EV-TRACK (transparent reporting and centralizing knowledge in extracellular vesicle research) platform has been recently launched to encourage the reporting of experimental parameters of EV-related studies to facilitate interpretation and reproducibility of the reported experiments (Consortium et al., 2017).

The two most common methods for isolating EVs from conditioned medium of cultured cells are differential centrifugation and density gradient centrifugation. While the differential centrifugation method isolates all EVs subtype, the density gradient centrifugation method isolates mainly exosomes; however, small size microvesicles can be also be part of EVs isolated by this technique. In this study, we isolated EVs using both differential centrifugation and density gradient centrifugation and we found p75NTR-containing EVs isolated by both methods are able to induce invasion of non-invasive glioma cells. These findings suggest that both p75NTR-containing exosomes and p75NTR-containing microvesicles are collectively able to induce glioma cell invasion.

Another caveat of investigating the role of EVs using current isolation methods is the presence of protein aggregates and small lipoprotein contaminants (Sodar et al., 2016). Although we found that p75NTR-containing EVs isolated by the two different isolation methods are able to induce glioma cell invasion, we cannot exclude the possibility of the presence of protein aggregates and lipoprotein contamination of our isolated EVs. In this aspect, we strongly believe that p75NTR-
containing EVs have a functional role in mediating glioma cell invasion since EVs isolated from cells expressing mutant p75\textsuperscript{NTR} receptor or EVs isolated from non-p75\textsuperscript{NTR} expressing cells did not have a dramatic impact on glioma cell invasion.

To examine invasion of glioma cells treated with EVs, we used the traswell invasion assay. In this assay, a thin layer of reconstituted Collagen I, one of the extracellular matrix (ECM) components that is found in brain and other organs, is coated onto the top of a porous membrane. The number of cells able to invade through Collagen I to the other side of the membrane are counted as a measure of invasive capability. Although this assay is reflective of the invasive ability of cells, it does not accurately represent the migration and invasion that occurs \textit{in vivo}. Specifically, it is not address the role of other ECM components in glioma cell invasion, or the interactions between the glioma cells and the normal brain cells in modulating glioma invasion. Therefore, results acquired by this assay may not necessarily reflect glioma cell invasion \textit{in vivo}. To overcome limitations of this \textit{in vitro} invasion assay, the invasive properties of the glioma cells are usually determined by their ability to form highly infiltrative tumors when implanted into the brains of SCID mice. In this study, to investigate the role of p75\textsuperscript{NTR}-containing EVs in inducing glioma cell invasion \textit{in vivo}, non-invasive glioma cells (U87-MG) were treated with equal amounts of EVs carrying the wild-type p75\textsuperscript{NTR} or the cleavage resistant p75\textsuperscript{NTR} form (p75Fas-S) for 24 hours \textit{in vitro}. An equal number of these cells or U87-MG control cells were then implanted into the brains of SCID mice. After 3 weeks, all the mice are still active and no sickness signs have been observed.

One of our future goals is determine if mRNA of p75\textsuperscript{NTR} is also transferred by EVs between glioma cells. Glioma cell-derived EVs containing mRNA are taken up by brain microvascular endothelial cells. By incorporating mRNA for a reporter protein into these EVs, EV-delivered
messages are translated by recipient cells (Skog et al., 2008). Subsequently, we want to determine whether p75\textsubscript{NTR} mRNA transported by glioma cell-derived EVs into a recipient cell will be translated by this cell.

Another area that needs to be investigated is whether p75\textsubscript{NTR} intracellular adaptor protein, PDLIM1, is also transported with p75\textsubscript{NTR} in EVs and whether it is essential for mediating glioma cell invasion by p75\textsubscript{NTR}-containg EVs. As discussed previously, PDLIM1 interacts with p75\textsubscript{NTR} at the PDZ-binding motif and is required for the p75\textsubscript{NTR} cell autonomous mechanism in mediating glioma invasion (Ahn et al., 2016). Downregulation of PDLIM1 or the complete loss of the p75\textsubscript{NTR} PDZ-binding motif ($\Delta$SPV) resulted in abrogation of invasion (Ahn et al., 2016). In the future, isolating EVs from cells lacking p75\textsubscript{NTR} PDZ-binding motif ($\Delta$SPV) and downregulated PDLIM1 cell and using those EVs in invasion assays will help determine the role, if any, of PDLIM1 in mediating glioma cell invasion by p75\textsubscript{NTR}-containg EVs.

5.3 Therapeutic implications of p75\textsubscript{NTR}

Identification of key regulatory proteins of GBM proliferation and invasion is extremely important clinically because this will be used to provide therapeutically relevant targets to inhibit GBM tumor progression and prevent tumor recurrence. Herein, we presented evidence that p75\textsubscript{NTR} is important in GBM proliferation and invasion, and the mere expression of p75\textsubscript{NTR} is sufficient to impart dramatic proliferative and invasive behaviors on genetically distinct GBM cells, therefore; p75\textsubscript{NTR} represents a potential target for both invasive and proliferative reservoirs in GBM. p75\textsubscript{NTR} has also been implicated in the progression of other tumors including melanoma, specifically in those tumors that metastasize to the brain, breast cancer, medulloblastoma, and head and neck squamous cell carcinoma. Therefore, therapies that target p75\textsubscript{NTR}, p75\textsubscript{NTR} downstream effectors,
or their ligands may not only be beneficial for GBM treatment, but they might be used to target other tumors.

Our observations that p75\textsuperscript{NTR} knockdown induced BTICs differentiation towards astrocytic lineages suggest that targeting p75\textsuperscript{NTR} might be used as a differentiation therapy for GBM. Differentiation therapy could be used to force BTICs to differentiate and lose their self-renewal property. Although inducing cancer cell differentiation is a rational therapeutic approach for targeting cancer cells, only few agents including retinoic acid (RA or Vitamin A), histone deacetylase inhibitors (HDAC) and bone morphogenetic proteins (BMPs), currently implicated in the differentiation therapy. The retinoic acid has been implicated in modulating cell differentiation and proliferation of acute promyelocytic leukemia (Massard, Deutsch, & Soria, 2006). RA treatment of BTICs induced morphology changes, induced growth arrest at G1/G0 to S transition, decreased cyclin D1 expression, induced the expression of lineage-specific differentiation markers Tuj1 and GFAP and reduced the expression of neural stem cell markers such as CD133, Msi-1, nestin and Sox-2 as well as inhibited their capacity to propagate subcutaneous and intracranial xenografts (Ying et al., 2011). Interestingly, most of these phenotypes were also observed in p75\textsuperscript{NTR} knockdown BTICs, suggesting that p75\textsuperscript{NTR} might be a potential target for differentiation therapy. BMP4 treatment of BTICs was also found to inhibit BTICs ability to form tumors and to induce their astrocytic differentiation, however, enrichment of SOX transcription factor-binding motifs in these astrocytes suggest that they are not fully differentiated cells and they might dedifferentiate to acquire their tumorigenic activity (Caren et al., 2015). Moreover, HDAC inhibited growth, induced differentiation, and apoptosis of BTICs \textit{in vitro} and \textit{in vivo} (P. Sun et al., 2009). Collectively, these findings highlight the importance of differentiation therapy of GBM, a therapy that may induced by targeting p75\textsuperscript{NTR} in BTICs.
Metabolic remodeling is a major phenotype in GBM cancer cells and refers to the alterations in the utilization and/or synthesis of several crucial metabolites including glucose, glutamine, glycogen, amino acids and fatty acids by cancer cells. Herein, p75\textsuperscript{NTR} knockdown was associated with major changes of cancer cell metabolite status including downregulation of pathways of fatty acids synthesis, amino acids metabolism, and changes in glycolysis and oxidative phosphorylation pathways. These p75\textsuperscript{NTR}-mediated metabolic changes could give several advantages for GBM diagnostic purposes. For instance, Positron Emission Tomography (PET) uses the increased glucose uptake by using 2-Deoxyglucose labeled with \(^{18}\text{F}\) radioemitter, to distinguish tumor regions from non-tumor or necrotic regions (Wolf, Agnihotri, & Guha, 2010). In addition, non-invasive MR techniques such as H-MR spectroscopy (MRS) of the brain also uses increased aerobic glycolysis by glioma cells to measure increases in choline and lactate levels with reduction of N-acetyl aspartate, the latter associated with normal neurons. Importantly, the MRS profile correlates glioma grades, with GBM having the highest choline and lactate levels (Wolf et al., 2010).

Regulation of immune cell activates within glioma microenvironment is another area that might be regulated directly or indirectly by p75\textsuperscript{NTR}. Recently, we showed that p75\textsuperscript{NTR} is associated with increased infiltration of macrophages/ microglia in tumor microenvironment (Alshehri et al., 2017). In this study, the microarray analysis of BT147 control and BT147p75\textsuperscript{NTR} showed that p75\textsuperscript{NTR} downregulation was associated with downregulation in gene expression patterns of several pathways associated with the inflammation and immune cell functions such as the inhibition of Toll-like receptors signaling (TLRs). TLR 2, 4 and 9 has been shown to be expressed by GBM cells and involved in tumor progression (Deng et al., 2014). The release of specific cytokines including IL-6, IL-8, and MCP-1 that protect cells from apoptosis or lead to enhance ability of
tumor cells to evade immune detection may also be due to the expression of TLRs on GBM cells (Deng et al., 2014). Therefore, targeting p75\textsuperscript{NTR} may help in recruiting and possibly re-educating immune cells within glioma microenvironment. Such therapeutic approaches have been recently used to target glioma cells and to inhibit tumor growth through crippling the colony stimulating factor-1 (CSF-1) signaling axis in the absence of chemotherapeutic drugs, which led to a decrease in tumor burden with an overall increase in survival using a specific mouse model of glioma (Pyonteck et al., 2013). Alternatively, microglia and tumor associated macrophages could be pharmacologically activated to secrete factors that suppress the growth of GBM cell \textit{in vitro} and significantly extend overall survival in orthotopic human glioma-bearing mice (Sarkar et al., 2014). In melanoma, a combined antibody therapy targeting melanoma cells that express p75\textsuperscript{NTR} and CD47, a transmembrane integrin-associated protein that involved in an anti-phagocytic signaling, resulted in a strong inhibition of tumor metastasis in patient-derived xenografts and associated with dramatic changes in the tumor and metastatic sites immune microenvironments (Ngo et al., 2016). These changes include a dramatic increase in the density of differentiated macrophages and a drastic decrease of inflammatory monocytes, pro-metastatic macrophages (CCR2+/VEGFR1+), and neutrophils, all of which are associated with disease progression (Ngo et al., 2016). All together, these findings suggest that p75\textsuperscript{NTR} might be a potential target to inhibit tumor growth and increase anti-tumorigenic immune cell populations not only in GBM but in other cancers.

Several inhibitors have been developed to reduce p75\textsuperscript{NTR} activity in CNS injuries and in various neurological diseases. For instance, the piperazine-derived compound, EVT901, has been shown to interfere with oligomerization of full-length p75\textsuperscript{NTR} in a dose-dependent manner, leading to a reduced neuronal death in the hippocampus and thalamus, reduced long-term cognitive
deficits, and reduced the occurrence of post-traumatic seizure activity in adult rats (Delbary-Gossart et al., 2016). Furthermore, The Longo and Massa laboratories used in silico screening to identify non-peptide small molecules that interacts specifically with p75<sup>NTR</sup> as ligands. These compounds were found to have a high specificity of p75<sup>NTR</sup> because they do not bind Trk receptors and were negative when screened for binding to >50 common G-protein coupled and ion channel receptors (Massa et al., 2006). One of these p75<sup>NTR</sup> specific compounds is, LM11A-31, which has more favorable advantages compared to other compounds. These advantages include suitable oral bioavailability and BBB penetration with a brain-to-plasma ratio >1, as well as the therapeutic efficacy of this compound both in vitro and in vivo models of multiple neurodegenerative disorders including AD and traumatic brain injury (Longo & Massa, 2013). Although using these inhibitors leading to the inhibition of p75<sup>NTR</sup>-induced cell death in multiple neurodegenerative disorders and traumatic brain injury, using them in GBM context might lead to inhibit tumor growth and invasion through eliminating p75<sup>NTR</sup>- expressing glioma cells.

The correlation between chemoresistant activity of cancer cells and p75<sup>NTR</sup> expression has been highlighted in different reports. For instance, gene set enrichment analysis of p75<sup>NT</sup>-expressing melanoma cells that have chemoresistant activities showed a strong association between p75<sup>NTR</sup> expression and genes involved in the regulation of DNA repair genes (Redmer et al., 2017). The downregulation of p75<sup>NTR</sup> in these cells decreased the expression of some DNA repair genes and partially increased sensitivity of these cells to chemotherapeutic agents (Redmer et al., 2017). In another study, p75<sup>NTR</sup> was found to promote melanoma cell survival and drive acquired resistance to the BRAF inhibitor vemurafenib, a highly effective treatment of melanoma (Lehraiki et al., 2015). Using in vivo selection model, our laboratory isolated different BTICs that acquired resistance to TMZ and showed that these cells restored their sensitivity to TMZ when
combined with disulfiram and copper (Lun et al., 2016). Interestingly, immunohistochemical analysis of p75\textsuperscript{NTR} in brain sections of mice bearing brain tumors generated by TMZ-resistance BTICs showed a strong expression of p75\textsuperscript{NTR} on these tumor cells compared to their TMZ-sensitive counterparts (Robbins lab, unpublished paper), suggesting that p75\textsuperscript{NTR} may contribute at the TMZ resistance in GBM tumors. Furthermore, we found p75\textsuperscript{NTR} is secreted from these cells through EVs; however, whether uptake of these EVs by TMZ-sensitive glioma cells is enough to mediate TMZ-resistance of these cells is still unknown. In agreement with this hypothesis, it has been recently shown that divergent evolution of TMZ resistance in BTICs is reflected in their EVs (Garnier et al., 2017). The mRNA expression profiles of individual tumors derived from the same isogenic BTIC line that expressed divergent and complex profiles of TMZ resistance markers, were recapitulated in the transcriptome of EVs released by these cells into the culture medium (Garnier et al., 2017). Together these data suggest that p75\textsuperscript{NTR} may contribute to the TMZ-resistance and EVs released by TMZ-resistant glioma cells reflect the divergent evolution of TMZ resistance in these cells.

In this thesis, we showed that p75\textsuperscript{NTR} is transferred between glioma cells through EVs to induce glioma cell invasion in recipient cells. In addition, p75\textsuperscript{NTR} was also found in EVs isolated from sera of mice bearing brain tumors generated by p75\textsuperscript{NTR}-expressing BTICs. Although EVs-containing potential diagnostic and prognostic markers, such as the mutant EGFR\textsubscript{vIII} (Skog et al., 2008), isocitrate dehydrogenase (IDH) mRNA (W. W. Chen et al., 2013) and elevated miR-21 levels (Akers, Ramakrishnan, et al., 2013) may represent great biomarkers that provide valuable information on GBM status and response to therapy, these EVs might also be used as a naturally therapeutic delivery tools. EVs represent an attractive tool of functional cargoes delivery to the brain for different reasons: EVs have a natural ability to shuttle biomolecules intercellularly, to
cross biological barriers such as BBB, to protect intraluminal contents and they could be isolated from a patient, loaded with therapeutic agents and reinjected into the same patient. Thus far, several molecules have been loaded in EVs including drugs, plasmid DNA, siRNA, miRNA, mRNA/protein, proteins, natural compounds, and viral particles (reviewed recently by Zappulli, Friis, Fitzpatrick, Maguire, & Breakefield, 2016). Interestingly, a zebrafish model of primary brain cancer was successfully treated by systemically administering murine brain endothelial cell derived-EVs loaded with doxorubicin and paclitaxel (T. Yang et al., 2015).

In conclusion, findings discussed in this section suggest that p75NTR can be a potential therapeutic target to modulate differentiation, metabolism and immune cells infiltration not only in GBM tumors and other tumors. Importantly, p75NTR activities in these tumors could be inhibited using small molecule inhibitors against p75NTR and these molecules and other therapeutic agents could be loaded in EVs and these vesicles can be used as a naturally therapeutic delivery tools.

5.4 Concluding remarks

In this thesis, we identified novel cell autonomous and cell non-autonomous roles of p75NTR in GBM progression. The cell autonomous roles of p75NTR include regulating sphere-forming capacity, proliferation, cell cycle progression, symmetric and asymmetric cell divisions, differentiation and invasion of genetically different patient-derived BTICs. Furthermore, knockdown of p75NTR in these BTICs inhibited their tumorigenic behaviors in vivo and extended the survival time of mice bearing brain tumors generated by p75NTR knockdown BTICs compared to their control counterparts. In addition to the cell autonomous roles of p75NTR in regulating GBM progression, this thesis provided novel findings about the cell non-autonomous roles of p75NTR in mediating glioma invasion. We showed that p75NTR is transported between glioma cells through EVs to induce glioma cell invasion in recipient cells. Interestingly, proteolytic processing of
p75NTR was detected in EVs and was required for meditating glioma cell invasion by p75NTR-containing EVs, as EVs isolated from glioma cells expressing a cleavage-resistant chimera of p75NTR (p75Fas-S) or from cells treated with a γ-secretase inhibitor failed to induce glioma cell invasion. p75NTR was also found in EVs isolated from sera of mice bearing brain tumors generated by p75NTR-expressing BTICs.

Indeed, further studies are required to determine if different p75NTR ligands, co-receptors, intracellular interactors and microenvironmental regulators are also involved in regulating both cell autonomous and cell non-autonomous roles of p75NTR in glioma progression. Accumulating evidence suggests that targeting the p75NTR signaling pathway may offer a new therapeutic strategy against treatment resistance and tumor recurrence. In general, one can consider the cell autonomous effects of signaling downstream of p75NTR as one signaling axis and the cell-non-autonomous effects of p75NTR signaling as another. Although targeting the p75NTR signaling pathway may provide some benefits to GBM patients, it may even have broader application for other cancers because p75NTR has also been implicated in cancers such as melanoma, breast cancer, medulloblastoma, and head and neck squamous cell carcinoma.
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