Netrins in glioma biology: regulators of tumor cell proliferation, motility and stemness

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred to by their roman numerals in the text

I.   Hu Y, **Ylivinkka I**, Chen P, Li L, Hautaniemi S, Nyman TA, Keski-Oja J, and Hyytiäinen M, Netrin-4 Promotes Glioblastoma Cell Proliferation through Integrin beta(4) Signaling. Neoplasia 14: 219-227, 2012

II.   **Ylivinkka I**, Hu Y, Chen P, Rantanen V, Hautaniemi S, Nyman TA, Keski-Oja J, and Hyytiäinen M, Netrin-1-induced activation of Notch signaling mediates glioblastoma cell invasion. J Cell Sci, 126: 2459-2469, 2013

III.   **Ylivinkka I** , Sihto H, Tynninen O, Hu Y, Laakso A, Kivisaari R, Laakkonen P, Keski-Oja J and Hyytiäinen, M, Motility of glioblastoma cells is driven by netrin-1 induced gain of stemness, J Exp Clin Canc Res, 9; 36(1):9, 2017

Publication I was included in the doctoral dissertation of Yizhou Hu (Netrin-4/integrin signaling in glioblastoma cells, University of Helsinki, 2016)

ABBREVIATIONS

2D	2-dimensional
3D	3-dimensional
ADAM	adamalysin related metalloproteinase
BrDU	Bromodeoxyuridine
CDK	cyclin dependent kinase
cDNA	complementary deoxyribonucleic acid
C. elegans	*Caenorhabtidis elegans*
CI	confidence interval
CNS	central nervous system
CSC	cancer stem cell
C-terminal	carboxy terminal
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butyl ester
DCC	deleted in colorectal cancer
D. melanogaster	Drosophila melanogaster
DMEM	Dulbecco's modified eagle medium
DSCAM	Down syndrome cell adhesion molecule
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EdU	5-ethynyl-2'-deoxyuridin
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FGF	fibroblast derived growth factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GBM	glioblastoma multiforme
GSC	glioblastoma stem-like cell
HA	hemagglutinin
HIF	hypoxia inducible factor
HR	hazard ratio
HSC	hematopoietic stem cell
IDH	isocitrate dehydrogenase
L1CAM	neural cell adhesion molecule 1
MGMT	O6-methylguanine-DNA methyltransferase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NetA	netrin A
NetB	netrin B
N-terminus	amino terminal
NTN1	netrin-1
NTN4	netrin-4
NICD	Notch intracellular domain
NOS	Not otherwise specified
NOTCH	Neurogenic locus notch homolog protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDAC	pancreatic ductal epithelial cell
PDGFRA	platelet derived growth factor receptor A
PFA	paraformaldehyde

PMEC	primary midgut epithelial cells
PTEN	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase
qRT-PCR	quantitative real-time polymerase chain reaction
RBP-JK	recombining binding protein suppressor of hairless
RNA	ribonucleic acid
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
SVZ	subventricular zone
TBS	Tris-buffered saline
TBP	TATA-binding protein
TCGA	The Cancer Genome Atlas
TERT	telomerase reverse transcriptase
TMZ	temozolomide
TP53	tumor suppressor protein 53
UNC5	uncoordinated 5
uPA	urokinase type plasminogen activator
WHO	World Health Organization
YAP	yes associated kinase

ABSTRACT

Glioblastoma is the most malignant brain cancer. Currently no cure has been established. The lethality of glioblastoma is a consequence of its extremely invasive nature: it rarely metastasizes outside the nervous system but effectively spreads throughout the brain parenchyma. This property, in addition to its challenging location makes it impossible to remove surgically. Furthermore, the tumors are heterogeneous and contain cells that are resistant to radiation therapy as well as to the only currently approved chemotherapy, temozolomide.

Netrins are secreted extracellular matrix proteins. They were initially identified as proteins essential for the correct axonal wiring of both vertebrates and invertebrates. Later, they were observed to regulate the branching morphogenesis of various organs including mammary gland, lungs and pancreas. During recent years increasing number of studies have linked netrins to various forms of cancer. For example, netrin-1 induces the invasion of pancreatic, colorectal and hepatocytic cancers and medulloblastoma and promotes the survival of breast and lung cancer and neuroblastoma. Netrin-4 can modulate tumor growth, angiogenesis, and metastasis.

In the current work, we analyzed how netrins contribute to glioblastoma growth. We discovered that both netrin-1 and -4 contribute to the malignancy of glioblastoma via independent pathways. Netrin-1 was upregulated in glioblastoma whereas netrin-4 was downregulated. However, both were associated with poor patient prognosis. The signaling pathways mediating the effects of these proteins were systematically explored.

First, our results revealed a new mechanism where netrin-4 controls glioma cell proliferation via UNC5B and ITGB4 receptors. During normal cell culture conditions netrin-4 is abundantly expressed, and it binds to both UNC5B and ITGB4 receptors that counteract each other and keep glioma cell proliferation in balance. However, during glioma progression netrin-4 expression is decreased and the signaling is shifted towards ITGB4. This led to increased cell proliferation and tumor growth.

Second, we discovered a mechanism through which netrin-1 promotes cell invasion. Netrin-1 expression associated with astrocytomas which are invasive glioma subtype. In glioblastoma cells it interacted with Notch2 and Jagged1 and facilitated the activation of the signaling. Subsequently, this led to an increase in cell invasion *in vitro* and *in vivo.* Furthermore, a unique invasion pattern was characterized where netrin-1 expressing cells were promoting the motility and stemness of the invasion leading stem-like cells.

Third, we designed and engineered a recombinant peptide that had the capacity to inhibit netrin-1 signaling. This peptide was able to overcome the effects of the full-length netrin-1 and specifically inhibited the invasiveness of the stem-like glioblastoma cells *in vitro* and *in vivo*. This peptide may prove out to be of therapeutic value in GBM treatment.

TIIVISTELMÄ

Glioomat ovat glia- eli hermotukisoluista alkunsa saavia aivokasvaimia. Glioblastooma on näistä syöpäkasvaimista pahanlaatuisin. Siihen ei toistaiseksi ole kehitetty hoitoa, ja potilaiden keskimääräinen elinaika on 15 kuukautta diagnosoinnin jälkeen. Glioblastooman pahanlaatuisuus johtuu kasvaimen syöpäsolujen aggressiivisesta leviämisestä terveen aivokudoksen sekaan. Tästä syystä se on lähes mahdoton poistaa kirurgisesti. Nämä syöpäsolut ovat myös sädehoidolle sekä toistaiseksi ainoalle hyväksytylle lääkehoidolle, temozolomidille, vastustuskykyisiä

Netriinit ovat erittyviä soluväliaineen proteiineja. Niiden on alun perin havaittu säätelevän aksonien kehitystä yksilönkehityksen aikana sekä selkärankaisissa että selkärangattomissa. Myöhemmin niiden on osoitettu säätelevän myös muiden haaroittuneiden elinten muodostumista. Viime vuosien aikana yhä useammat tutkimukset ovat osoittaneet netriinien olevan tärkeässä osassa myös syöpäbiologiassa: netriini-1 lisää useiden eri syöpätyyppien leviämistä sekä syöpäsolujen selviytymistä, ja netriini-4 puolestaan on yhdistetty syöpäkasvainten kykyyn kasvattaa verisuonia sekä niiden kykyyn lähettää etäpesäkkeitä.

Tässä väitöskirjatyössä on tutkittu netriinien vaikutuksia glioblastoomaan. Tutkimuksissa osoitettiin, että lisääntynyt netriini-1:n tuotto ja vastakohtaisesti vähentynyt netriini-4:n tuotto ovat yhteydessä potilaiden huonompaan ennusteeseen. Signalointireittejä, joiden kautta nämä proteiinit vaikuttavat syöpäsoluihin, seulottiin systemaattisesti. Tutkimuksessa havaittiin, että kumpikin näistä proteiineista vaikuttaa glioblastoomasoluihin itsenäisesti eri signaloittireittien välityksellä. Netriini-4:n osoitettiin olevan tärkeä glioblastoomasolujen jakautumisen säätelijä ja sen tuoton vähentyminen mahdollisti kontrolloimattoman solujen jakautumisen ja täten kasvaimen kasvun. Netriini-1:n puolestaan osoitettiin olevan merkittävä glioblastoomasolujen liikkuvuuden säätelijä. Sen lisääntynyt tuotto sai aikaan syöpäsolujen muuttumisen kantasolumaisemmiksi ja aggressiivisemmin terveen aivokudoksen sekaan leviäviksi.

Lisäksi tässä työssä suunniteltiin ja rakennettiin peptidi, joka pystyi estämään netriini-1:n vaikutukset glioblastoomasoluissa. Koska tämän peptidin tuotto vähensi glioblastoomasolujen kantasolumaisuutta sekä niiden kykyä levitä terveen aivokudoksen sekaan, voi sillä tulevaisuudessa olla potentiaalia glioblastooman lääkehoidon kehityksessä.

INTRODUCTION

Central nervous system (CNS) consists of two types of cells: neurons and glial cells. While neurons are the main cells that process and mediate information the glial cells provide many supportive functions to them. Glial cells can be further divided into macro- and microglial cells (reviewed in Sierra *et al.*, 2016). Microglial cells are specialized immune cells of the CNS (Gehrmann *et al.*, 1995). They are a special group of macrophages that are responsible for the immune defense of the CNS. Within the human brain, three types of macroglial cells are present: astrocytes, oligodendrocytes and ependymal cells. The main function of oligodendrocytes is to isolate the neurons: they wrap around the neurons and provide isolating myelin sheath for them (Baumann and Pham-Dinh, 2001). Astrocytes are branched cells which are in contact with neurons, each other and blood vessels (Nag, 2011). They provide many supporting functions for neurons for example by up taking excess potassium ions that are produced during synaptic emission or by secreting many growth factors that are essential for the well-being of neurons (Krencik *et al.*, 2016). In addition, astrocytes participate in the development of blood brain barrier (Haseloff *et al.*, 2005). Ependymal cells are specialized cells that create the linings of the ventricles of the brain and the central canal of the spinal cord. Furthermore, they contribute to the production of cerebrospinal fluid.

Diffuse gliomas are tumors of macroglial cells within the brain. They are the most common primary brain tumors in humans (Maher *et al.*, 2001; Ostrom *et al.*, 2015). Approximately 400 gliomas are diagnosed in Finland per year (Maenpaa, 2010). Currently the cell of origin of gliomas is unknown but it has been proposed that the tumors arise from mutated mature astrocyte or oligodendrocyte or undifferentiated neural stem or precursor cell. Gliomas are graded into four grades on the basis of their malignancy. Most aggressive form of these tumors, glioblastoma is a devastating disease which has remained incurable despite numerous treatment attempts during recent years. The lifetime expectancy of patients is only 15 months after diagnosis. The lethality of glioblastoma is a result of its highly heterogenous and infiltrative phenotype (Maher *et al.*, 2001). The tumors are impossible to remove surgically because of aggressively, throughout the brain invading tumor cells. In addition, the glioblastoma cells are extremely resistant to radiation and chemotherapy (Raizer and Parsa, 2015). Therefore, better understanding of the biology of the glioblastoma cells is needed in hopes of invention of new therapeutic options.

Netrins are secreted proteins that are essential for the development of both vertebrates and invertebrates. They act as chemoattractants and regulate both cell polarization and migration during axon guidance, vascular patterning and branching morphogenesis of different organs (Cirulli and Yebra, 2007). Within recent years netrins have also been linked to tumorigenesis of various epithelial and nervous system cancers including neuroblastoma, colorectal, breast and lung cancer (Ko *et al.*, 2014). They have been shown to regulate cancer cell migration and survival.

In this thesis the role of netrins in human glioblastoma tumors was assessed. The role of both netrin-1 and netrin-4 was investigated in different aspects of glioma biology, including cancer cell proliferation, survival, invasion and stemness. In addition, the signaling pathways mediating the effects of netrins were screened systematically. The findings help us to better understand the molecular mechanisms behind the disease and guide our way to the development of new treatment options.

REVIEW OF THE LITERATURE

1. Human gliomas

1.1. Classification of gliomas

Gliomas are the most common human primary brain tumors. They are tumors of the macroglial cells, mainly astrocytes and oligodendrocytes. Gliomas often emerge in the frontal lobe area and are slightly more common in men than in women (Cancer Genome Atlas Research Network *et al.*, 2015). The classification of gliomas has been based on histological characterization of the tumors according to the guidelines set by the World Health Organization (WHO) (Louis *et al.*, 2007). Until this year gliomas were divided into three major classes: astrocytoma, oligoastrocytoma and oligodendroglioma, based solely on their histopathological status of glial cell differentiation (Louis *et al.*, 2007; Maher *et al.*, 2001). According to this classification astrocytomas were divided into grade II-IV tumors whereas oligoastrocytoma and oligodendrogliomas were typically grade II tumors.

Within recent years, excessive efforts have been made to investigate the genetic alterations of gliomas. These advances have revealed distinct mutations in different glioma subtypes. Based on these findings WHO has published new guidelines for the treatment and classification of these tumors (Louis *et al.*, 2016). According to the current view gliomas emerge from a common precursor cell. Therefore previous glioma classes, astrocytomas, oligoastrocytomas and oligodendrogliomas have been combined under one group named diffuse gliomas. This major group is then further divided into subgroups based on the different mutations acquired by the tumors (figure 1). Previous oligoastrocytoma class has been completely excluded from the new classification since these tumors can now be included into the new subtypes based on the mutational status. This simplifies the classification of oligoastrocytic tumors that present characteristics of both astrocytomas and olidendrogliomas on the cellular level.

According to the current view, mutation in isocitrate dehydrogenase (IDH) gene is the earliest mutation on the progression of gliomas (Balss *et al.*, 2008; Sanson *et al.*, 2009). This mutation is observed in 80% of the low grade diffuse gliomas and it is common in both astrocytic and oligodendrocytic tumors. The mutation occurs more frequently in IDH1 gene but also mutations in IDH2 gene have been detected. If additional co-deletion of chromosome arms 1p and 19q is detected tumors are categorized as grade II or III oligodendrogliomas (Louis *et al.*, 2016). If a mutation in the tumor suppressor protein 53 (TP53) and transcription regulator ATRX (ATRX) are detected the tumors are diagnosed as diffuse astrocytoma grade II. Furthermore, loss of chromosome arms 9p and 10q are commonly observed in anaplastic astrocytoma grade III and secondary glioblastoma (GBM) grade IV, respectively.

The division of the diffuse gliomas into mutational subtypes improves the diagnosis and the prediction of patient response to treatment. The prognosis of oligodendroglioma patients is better than astrocytoma patients with median survival times 8.0 years and 6.3 years respectively (Cancer Genome Atlas Research Network *et al.*, 2015). The median age of patients diagnosed with grade II or III glioma is 42.6 years at the time of diagnosis and does not significantly vary between the subtypes (Cancer Genome Atlas Research Network *et al.*, 2015).

Precursor cell

20% — IDH wildtype
80% — IDH mutated

IDH wildtype → Diffuse astrocytoma, IDH WT / Oligodendroglioma, NOS

IDH mutated:
30% → 1p/19q deletion → Oligodendroglioma grade II/III
50% → ATRX loss / TP53 mutation → Diffuse astrocytoma grade III → 9p loss → Anaplastic astrocytoma grade III → 10q loss → 10% → Secondary glioblastoma grade IV

IDH wildtype
10q loss
PTEN mutation
EGFR amplification
CDKN 2A/B deletion
Tp53 mutation
TERT promoter mutation
→ 90% → Primary glioblastoma grade IV

Figure 1. Classification of human gliomas. Schematic presentation of the progression and current classification of diffuse gliomas. White boxes depict gained mutations and grey boxes the tumor classification. See explanation for abbreviations on page 8-9.

1.2. Glioblastoma

Among gliomas, glioblastoma multiforme (GBM) is the most malignant tumor. They emerge either as primary tumors without previous clinical manifestations or, as described above, as secondary tumors that rise from a lower grade gliomas (figure 1). The majority, approximately 90% of the GBMs are primary tumors and 10% progress from diffuse astrocytoma within 5 years (Ohgaki and Kleihues, 2013). Secondary GBMs are often located in the frontal lobe whereas primary GBMs have no location specificity (Lai *et al.*, 2011). The median age at the time of diagnosis is 45 years for secondary GBM patients and 60 years for primary GBM patients (Ohgaki and Kleihues, 2013). The expected lifetime of patients after diagnosis is 15 months for primary GBM and 31 months for secondary GBM (Louis *et al.*, 2016).

The histology of primary and secondary GBM is similar. However, the accumulated mutations vary from each other. Primary glioblastomas carry wildtype IDH whereas the majority of secondary glioblastomas have mutated IDH. Since secondary GBMs have developed from the low grade astrocytomas they also harbor mutations in the TP53 and ATRX. Primary GBMs commonly have amplification of epidermal growth factor receptor (EGFR), mutated phosphatase and tensin homolog (PTEN), loss of whole chromosome 10, deletion in cyclin

dependent kinase inhibitor 2 (CDKN2) and mutated telomerase reverse transcriptase (TERT) promoter (Ohgaki *et al.*, 2004).

Several other less frequent genetic aberrations have been detected in GBM tumors. Based on these there have been attempts to divide the GBMs into 3-4 molecular subtypes (Brennan *et al.*, 2013; Verhaak *et al.*, 2010). The subtypes include classical, proneural, neural and mesenchymal type GBM. While the majority of secondary GBMs belong to proneural subtype, primary GBMs can belong to any of them. Classical subtype is characterized by EGFR amplification (97%) and CDKN2 deletion, proneural with IDH and TP53 mutations and PDGFRA amplifications, mesenchymal GBMs frequently have NF-1 mutation and MET amplification (Phillips *et al.*, 2013; Verhaak *et al.*, 2010). These subtypes may be of clinical value if personalized treatments will be available for GBM. However, the GBM tumors may be even more heterogeneous, which may complicate the classification. When one biopsy of GBM tumor was analyzed on single cell RNA sequencing it was revealed that individual cells among the biopsy may belong to different tumor subtype (Patel *et al.*, 2014). Furthermore, the copy number alterations differ within several samples collected from various locations around same tumor (Sottoriva *et al.*, 2013). Therefore, individual GBM cells display great heterogeneity within each tumor. One biopsy taken during the operation may not necessarily properly characterize the tumor and may lead to incorrect GBM subtype classifications. Especially this aspect should be considered if personalized GBM treatment will be applied in the future.

1.2.1. Hallmarks of glioblastoma

The histopathological features of GBM tumors are infiltrative growth within the healthy brain tissue, necrotic tumor core which is surrounded by dense cell areas called pseudopalisade structures and excessive angiogenesis resulting in abnormal tumor vasculature called glomerular vessels (Brat *et al.*, 2004a; Rojiani and Dorovini-Zis, 1996). Especially the pseudopalisade structures and the glomerular vessels distinguish GBM tumors from the lower grade II and III astrocytomas and oligodendrogliomas. It is suggested that hypoxia within the tumor core is the cause of these GBM hallmarks. Hypoxia upregulates a multifunctional protein called hypoxia inducible factor (HIF) in the tumor cells. HIF regulates several signaling pathways that are linked to cell metabolism, motility, angiogenesis and stemness of the tumor cells (reviewed in (Rankin and Giaccia, 2016)). The pseudopalisade structures are shown to be hypoxic and to consist of actively migrating cells (Brat *et al.*, 2004b). Furthermore, HIF can upregulate the production of VEGF within the tumors which leads to excess endothelial cell proliferation. However, the vessels formed are tortuous and leaky which results in increased interstitial pressure and cancer cell evasion from the primary tumor (Jain, 2013). This causes a cycle where the lack of nutrients and oxygen within the tumor core forces the tumor cells to disseminate and invade into the brain parenchyma. This infiltrative phenotype makes it impossible for the GBM to be completely resected with surgery.

1.2.1.1. Invasive modes of glioblastoma cells

GBM cells use neuron networks and white matter tracts as highways to spread throughout the brain (figure 2). In addition, they actively co-opt the brain vessels, attach to the vascular

basement membrane and move along the perivascular space. Despite the active spreading within the brain parenchyma, GBM cells extremely rarely metastasize to other organs.

The molecular mechanisms driving the motility of GMB cells have been widely investigated during recent years. The major barrier that the tumor cells encounter while leaving the primary tumor is the extracellular matrix (ECM) of the brain. It consists mainly of hyaluronic acid, proteoglycans and tenascin glycoproteins (Novak and Kaye, 2000). Due to the lack of laminin, fibronectin or collagens it is relatively soft matrix and less fibrous and cross-linked than the ECM in many other organs. The vascular basement membrane that the GBM cells also use as a migratory platform contains fibronectin, laminin and collagens III and IV.

Cells attach to the ECM via specialized adhesion receptors. The range of these receptors overexpressed in GBM tissue include integrin receptors α3β1, α2β1, α5β1, α6β1, α6β4, α9β1, αvβ3 and αvβ5 that bind various ECM proteins including laminin, tenascins, fibronectin and vitronectin (Gingras *et al.*, 1995; Paulus *et al.*, 1993). In addition, hyaluronan receptors CD44, ICAM-1 and L1-CAM are upregulated in GBM compared to normal brain (Gingras *et al.*, 1995; Paulus *et al.*, 1993). Via these receptors the GBM cells can attach to the surrounding matrixes and migrate away from the primary tumor site. In addition, GBM cells express various proteases that can cleave the ECM and thereby aid the motility of the cells. The main proteases expressed by are ADAMs, uPA, MMPs and cathepsins (Nakada *et al.*, 1999; Rempel *et al.*, 1994; Rooprai *et al.*, 1998; Wildeboer *et al.*, 2006; Yamamoto *et al.*, 1994). While the cancer cells are disseminating from the primary tumor the proteases play an important role in detaching the invasive cells from the tumor mass.
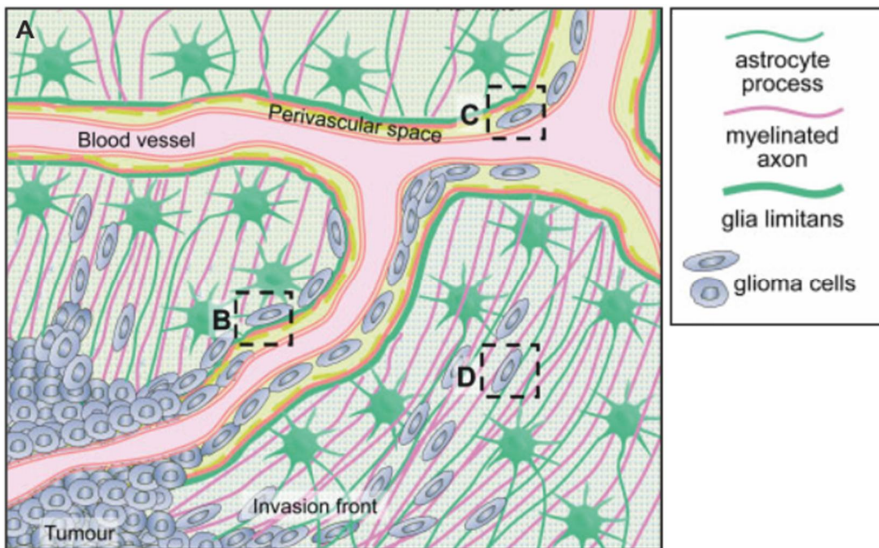

Figure 2. GBM cell invasion. (A) Schematic presentation of GBM cells disseminating from the primary tumor. GBM cells can either invade along (B) the border of stroma and blood vessels, (C) within the perivascular space of blood vessels or (D) along the white matter tracts. Modified from (Gritsenko *et al.*, 2012)

Cancer cells can invade as single cells or collectively and, the invasion mode can be amoeboid or mesenchymal like (Clark and Vignjevic, 2015; Friedl and Wolf, 2010). Mesenchymal invasion is dependent on integrins and proteases while the amoeboid like invasion is mediated by Rho/Rac signaling and actin remodeling which allows the cells to squeeze through soft matrixes. In general, stiffer ECM is proinvasive (Kai *et al.*, 2016). The rigidity provides more adhesion sites to the cells and the support allows them to use mechanical force to move forward.

The composition of the brain ECM suggests that the GBM cells utilize amoeboid type invasion. However, the variety of adhesion receptors and proteases expressed by the GBM cells indicates that they favor mesenchymal type invasion. Interestingly, depending on the matrix GBM cells are capable of both invasion types *in vitro* (Herrera-Perez *et al.*, 2015). GBM cells implanted into rat brain showed more Rho/Rac activity upon invasion along the white matter tracts than when moving within the perivascular space (Hirata *et al.*, 2012). These findings suggest that the GBM cells are plastic and can adjust their invasive mode according to their surroundings. Furthermore, the GBM cells themselves modulate the surrounding ECM. They secrete fibronectin, laminin and collagens which make the ECM stiffer and support their invasive phenotype (Bjerkvig *et al.*, 1989; Mahesparan *et al.*, 2003).

Recently, it was observed that the distant, invasive GBM cells form a functional network. The tumor cells are in contact within each other via long, actin rich membrane tubes termed tumor microtubes (TM) (Osswald *et al.*, 2015). TMs allow the tumor cells to communicate within each other despite the distance. For example, cells were able to send calcium signaling waves via TMs. Furthermore, when part of the tumor was exposed to radiation and cells had died, distant cells could use the TMs to travel to the site of damage and replace the dead cells. Based on these findings GBM tumors are very plastic and dynamic entities.

1.2.2. Current standard care of glioblastoma

The current treatment options of newly diagnosed and secondary glioblastoma rely heavily on surgical resection and radiation therapy of the tumor. The surgical resection of GBM is aimed to perform as aggressively as possible. To locate the tumor and map the functional brain regions functional magnetic resonance imaging and computational tomography are performed before the surgery (Raizer and Parsa, 2015). During the operation Gliolan (5-aminolevulinic acid labeling) is administered to the patients. This fluorescent dye accumulates into the tumor cells allowing them to be visually detected during the operation. If the tumor is located near functional brain regions the craniotomy can be performed to awake patients (Raizer and Parsa, 2015). This allows the careful mapping of for example speech areas during the operation. After the surgery the patient is given concomitant radiation and chemotherapy. Currently only one drug, temozolomide (TMZ), is approved for the standard care of newly diagnosed glioblastoma.

However, due to the infiltrative growth the surgical resection is nearly always incomplete. The remaining tumor cells are capable of growing a new tumor and lead to recurrent glioblastoma for which there are no common treatment guidelines. Surgical resection and TMZ are often utilized. In addition, several targeted therapies have been investigated within recent years, which will be discussed below.

1.2.2.1. Temozolomide therapy

TMZ was discovered in 1980s (reviewed in (Friedman *et al.*, 2000)). Its effect is based on its capability to alkylate DNA in different positions. The most toxic methylation occurs in the O6-guanine position. This leads to defects in translation of DNA, subsequent cell cycle arrest and apoptotic death of the cancer cells. TMZ has been shown to effectively kill glioblastoma cells in mice xenograft studies (Friedman *et al.*, 1995; Plowman *et al.*, 1994). In phase III clinical trial (EORTC-NCIC trial) containing 573 high-grade glioma patients concomitant radiation and TMZ treatment after first surgery prolonged median patient survival from 12,1 months to 14,6 months (Stupp *et al.*, 2005). However, the treatment did not cure the patients but offered only prolonged survival. Within a five years follow-up time most of the patient had had a recurrent tumor and died of the disease (Stupp *et al.*, 2009). The major side-effects of TMZ treatment were grade 3 or 4 hematologic toxic effects which were observed in seven percent of patients (Stupp *et al.*, 2005).

In approximately 55% of the glioma patients TMZ treatment showed no effect. This is due to active O6-methylguanine-DNA methyltransferase (MGMT) enzyme in the tumor cells. MGMT is an excision repair enzyme which removes the TMZ induced O6-guanine methylation and thereby prevents the TMZ induced cell death. However, in some patients the MGMT enzyme is not expressed because of epigenetic silencing of the MGMT promoter regions. This patient group responds well to TMZ treatment (Stupp *et al.*, 2005). The genetic testing for the MGMT status of the patients allows the targeting of the treatment. This is important especially for the elderly patients. However, the MGMT status may have potential prognostic value only among the patients with classical GBM subtype (Brennan *et al.*, 2013).

1.2.2.2. Targeted therapies

Because of the large number of patients that do not respond to TMZ therapy, new therapeutic applications are needed. One of the hallmarks of glioblastoma is excessive angiogenesis in the tumors. Therefore, anti-angiogenic therapy is a possible therapeutic option for glioblastoma. Bevacizumab is an antibody which neutralizes the vascular endothelial growth factor A and thus inhibits the signaling through vascular endothelial growth factor receptor (Presta *et al.*, 1997). This reduces the angiogenesis in the tumors and thereby limits tumor's oxygen supply and growth. Bevacizumab has been effective in animal models of several cancers including glioblastoma (Ferrara *et al.*, 2004; Jahnke *et al.*, 2009; Mathieu *et al.*, 2008) . In addition, it has been proven effective in two phase I clinical trials that included patients with advanced solid tumors including colon, renal, lung, breast and cervical cancer (Gordon *et al.*, 2001; Margolin *et al.*, 2001).

Because of the promising treatment results in other cancers it was considered for glioblastoma therapy too. So far several phase II trials administering bevacizumab with TMZ or other therapeutics to patients with recurrent GBM have been conducted (reviewed in (Field *et al.*, 2015; Niyazi *et al.*, 2016; Seystahl *et al.*, 2016; Venur *et al.*, 2015)). Majority of the studies administered bevacizumab in combination with irinotecan. A slight prolongation of progression free survival was observed in these studies. However, the majority of them included only small patient cohort and did not design the case controls properly. Due to these shortcomings bevacizumab has not been approved as a GBM therapy in Europe. In contrast, bevacizumab has been approved as standard care for recurrent glioblastomas in United States. This decision was especially encouraged by the increase in overall survival (9,2

months in bevacizumab treated vs 8,7 months in control group) observed in one study (Friedman *et al.*, 2009).

To further evaluate the efficacy of bevacizumab in newly diagnosed GBM, two large, properly case controlled trials were recently conducted (Chinot *et al.*, 2014; Gilbert *et al.*, 2014). In these trials the bevacizumab treatment was combined with standard TMZ treatment. In neither of the trials the pre-set criteria for successful study were met. A subtle increase in progression free survival was observed but the overall survival was not improved. It has been suggested that the bevacizumab treatment may alter the glioblastoma phenotype into more invasive which gives an advantage for the tumor cells to escape the therapy (Baker *et al.*, 2014). In addition, the invasive tumor type is more difficult to locate in MRI and may result in false prolongation of progression free survival. Moreover, the tumor cells might adapt to the hypoxic tumor environment and change their metabolism towards glycolytic cycle and thus gain resistance to bevacizumab (Fack *et al.*, 2015).

In addition to bevacizumab, targeting of the integrins and EGF receptor have been investigated as treatment options for GBM (reviewed in (Chen *et al.*, 2016)). However, neither of these have been successful so far. More studies need to be conducted to fully understand the consequences and benefits of the targeted therapies in glioblastoma. In future trials, the increased knowledge of the molecular subclasses of GBM should be considered and may lead to more personalized treatment options.

2. Glioblastoma stem cells

2.1. Cancer stem cell hypothesis

Normal tissues contain a population of undifferentiated stem cells that are capable to differentiate into diverse cell types within an organism. In normal conditions, the stem cells divide asymmetrically which means that they give rice to one stem cell daughter cell and one differentiated daughter cell (Morrison and Kimble, 2006). This way they can maintain the stem cell number as constant while still providing new differentiated cells for the tissue. This is a key mechanism to regenerate tissues.

The cancer stem cell hypothesis originates from the investigation of hematological malignancies. It assumes that cancers consist of two types of tumor cells: stem-like cells and differentiated, bulk tumor cells. Similarly as normal stem cells in tissues, the population of the stem-like cancer cells is smaller but they are responsible for maintaining the tumor whereas the bulk tumor cells make up the most of the tumor volume. Acute myeloid leukemia initiates when genetic aberrations occur in the hematopoietic stem cells (HSC) or committed progenitor cells increasing their self-renewing capability (Bonnet and Dick, 1997). The transformed HSCs divide according to the hierarchical cell division mode of the normal HSCs but cannot give rise to normal progeny. Instead, they give rise to tumorigenic progenitor cells and thereby maintain the leukemia (Bonnet and Dick, 1997). Later on, similar observations of oncogenic stem cells have been made in solid tumors including breast, colon, pancreatic and lung cancers (reviewed in (O'Brien *et al.*, 2009)). These mutated tumor maintaining cells are termed cancer stem cells (CSC) (Clarke *et al.*, 2006). They share similarities with their stem cell counterparts of normal tissues. Their most important features are their ability to self-renew, to differentiate into non-tumorigenic cancer cells, to

phenocopy the original tumor of the patient and the capability to initiate tumor growth in rodents (Clarke *et al.*, 2006).

2.2. Discovery and characterization of glioblastoma stem cells

Like other solid tumors also GBMs are heterogeneous and contain populations of differentiated tumor cells and cells harboring similarities with neural stem cells. The nomenclature of the CSC population in GBMs has not been standardized. These cells are often called glioblastoma stem-like cells, glioma initiating cells, tumor propagating cells, glioma stem cells and glioblastoma stem cells. Here this cell population is referred to as glioblastoma stem cells (GSCs).

The first indication of GSCs was discovered in cultured human and rodent GBM cells when they were detected to be expressing neural stem cells markers nestin and CD133 under *in vitro* culture conditions (Ignatova *et al.*, 2002; 2002; Kondo *et al.*, 2004; Singh *et al.*, 2003). These cells were also capable of self-renewing. Next, GSCs were isolated from patient-derived brain tumors simultaneously in two independent studies. One study isolated GSCs based on their expression of CD133 (Singh *et al.*, 2004). It was observed that already 100 CD133-positive cells were able to initiate brain tumor when implanted into mice in contrast to 100 000 CD133-negative cells that did not initiate tumors at all. Furthermore, the xenografted tumors successfully recapitulated the characteristics of the original human tumor. In the other study human primary brain tumor cells were cultured *in vitro* under conditions that were known to maintain neural stem cells (Galli *et al.*, 2004). Isolated tumor cells were then transplanted into mice where they effectively formed tumors that recapitulated GBM hallmarks. In addition, the cells were able to differentiate into astrocyte-like, neuron-like and oligodendrocyte-like tumor cells *in vitro.* Serial transplantation of these cells also proved their ability to self-renew and maintain the tumor.

After the first studies identifying the GSC populations within GBM tumors, numerous studies have tried to further characterize these cells. The neurosphere culture system where cells are kept under serum free conditions supplemented with recombinant EGF and FGF has been widely accepted method for passaging of the GSCs (De Witt Hamer *et al.*, 2008; Lee *et al.*, 2006). Using this culture system the GSCs can be derived from fresh GBM biopsies (Hasselbach *et al.*, 2014a). In addition, laminin coated cell culture plates have been suggested to be a reliable method for adherent GSC culture conditions (Pollard *et al.*, 2009). Using rat GBM models it has been proposed that the long-cultured cell-lines also contain a small population of GSCs and could be used as a source for the GSCs (Kondo *et al.*, 2004). However, this observation was later challenged because the cell-line derived GSCs do not resemble the characteristics of GSCs freshly isolated from the patient tumors (Ahmad *et al.*, 2014; De Witt Hamer *et al.*, 2008). Therefore, freshly isolated cells are thought to be a better source of GSCs.

Neural stem cells reside in a specialized niche in the subventricular zone and hippocampus in the adult mammalian brain (Doetsch *et al.*, 1999; Johansson *et al.*, 1999). The niche provides favorable growth conditions for the stem cells. Within the niche the stem cells are nurtured and kept under undifferentiated state. The neural stem cells are often in contact with the microvessels of the niche. These vessels lack the blood-brain barrier and thus are able provide nutrients and growth factors to the stem cells (Shen *et al.*, 2008; Shen *et al.*, 2004; Tavazoie *et al.*, 2008). Furthermore, the ability to grow under hypoxic condition is a

typical feature of the stem cells. In human brain the oxygen concentration varies from 0.55% in the midbrain to 14% on the surface of the brain (De Filippis and Delia, 2011). Since the neural stem cell niche locates deep inside the brain it is relatively hypoxic. The hypoxia activates pathways that regulates the maintenance and self-renewal of the neural stem cells (Gustafsson *et al.*, 2005; Hu *et al.*, 2011).

The GSCs have been observed in various locations within the GBM tumors suggesting that there may be different niches for them. Like the neural stem cells, also the GSCs reside in hypoxic regions within the GBM tumors. In fact, hypoxia is an important factor responsible for the maintenance of the GSCs. It promotes the self-renewal and maintenance of the GSCs and even reprograms the bulk tumor cells into more stem-like state (Bar *et al.*, 2010; Heddleston *et al.*, 2009; Inukai *et al.*, 2015). The response for hypoxia may be regulated by both HIF1α and HIF2α proteins. HIF1α expression was upregulated in majority of GBM cells in the hypoxic regions (Li *et al.*, 2009; Soeda *et al.*, 2009) whereas HIF2α was upregulated specifically in CD133-positive GSCs (Li *et al.*, 2009). HIF2α was upregulated in the GSCs even upon minor changes in the oxygen balance whereas HIF1α upregulation was observed only under severely hypoxic conditions (Li *et al.*, 2009). Interestingly, silencing of HIF2α reduced GSC ability to initiate tumors in mice (Li *et al.*, 2009) suggesting that hypoxia may be a critical factor for the growth of the GSCs. Furthermore, this interplay of various HIF proteins may give advantage for the GSCs to survive within the tumor core.

Paradoxically, GSCs reside also on perivascular niches next to tumor microvasculature (Calabrese *et al.*, 2007; Charles *et al.*, 2010; Lathia *et al.*, 2010). In normal tissues the blood vessels are the source for oxygen and maintain the correct tissue homeostasis. Therefore, it is possible that the tumor vessels provide oxygen or other nutrients to GSCs similarly to the normal neural stem cells. However, the abnormal vasculature of the GBM does not function properly. Despite the excessive tumor angiogenesis the GBM tissue remains hypoxic or even anoxic (Rampling *et al.*, 1994). Therefore, the proximity to vasculature may offer other benefits for the GSCs. Indeed, the endothelial cells were observed to produce nitric oxide that promoted the maintenance and self-renewal of the GSCs via activating Notch pathway (Charles *et al.*, 2010). *In vivo*, inhibition of the nitric oxide production inhibited tumor growth in spontaneous glioma mouse model (Charles *et al.*, 2010). Furthermore, endothelial cells also expressed Notch ligands that activate the pathway leading to increased self-renewal of GSCs (Zhu *et al.*, 2011).

2.3. Contribution of glioblastoma stem cells to the tumor malignancy

The GSCs have been connected to several aspects that contribute to the lethality of GBM. Because the GSCs show enhanced capability to give rise to brain tumors in mice even when implanted in very low numbers (Singh *et al.*, 2004), they are thought to be the GBM initiating cells. Furthermore, somatic mutations introduced to neural stem cells are enough to induce spontaneous brain tumor formation in mice (Alcantara Llaguno *et al.*, 2009).

The GSCs exhibit increased resistance to radiation therapy. They rapidly activate the DNA damage checkpoint after radiation therapy via L1-CAM mediated activation of c-MYC (Bao *et al.*, 2006a; Cheng *et al.*, 2011b). In addition, they simultaneously activate the G2-M cell-cycle checkpoint after the exposure to the radiation (Ahmed *et al.*, 2015). This gives them advantage to avoid the radiation induced cell death. Therefore, the concordant inhibition of cell-cycle checkpoint and DNA repair machinery may sensitize the GSCs to radiation therapy

(Ahmed *et al.*, 2015). Furthermore, the GSCs show increased resistant towards TMZ chemotherapy. This has been investigated utilizing a genetic glioma mouse model where neural stem cells were labeled with nestin-GFP transgene and bred with genetically engineered mouse model that harbors mutations in p53, NF1 and PTEN (Chen *et al.*, 2012). These mice spontaneously form astrocytic tumors with full penetrance (Alcantara Llaguno *et al.*, 2009; Chen *et al.*, 2012). Interestingly, the tumors that arose contained two type of cells: proliferative, GFP-negative tumor cells and stem cell like, non-proliferative, GFP-positive cells. When TMZ was administered to the mice the GPF-negative cells died while GFP-positive cells persisted and even gave rise to new tumors (Chen *et al.*, 2012). This suggests that the GSCs are resistant to the TMZ-therapy possibly due to their slow proliferation rate. Moreover, via their capability to give rise to tumorous progeny they may cause the tumor relapse.

The normal neural stem cells are typically passive cells that remain in their niche under normal conditions (Kazanis, 2012). However, during pathological conditions they actively migrate to the sites of injury (Kazanis, 2012; Kojima *et al.*, 2010). Similarly, the GSCs show increased potential for motility. When GSCs were implanted into mice brain they produced more invasive tumors than their non-stem-like counterparts (Cheng *et al.*, 2011a). In human tumor biopsies and xenograft models the GSCs have been located to the invasive tumor front suggesting that they play a role in the spreading of the tumor (Cheng *et al.*, 2011a; Kitai *et al.*, 2010). Furthermore, they expressed various proteases that are known to facilitate cell migration (Cheng *et al.*, 2011a). *In vitro* upregulation of nestin has indeed been connected to enhanced invasive potential of glioma cells (Ishiwata *et al.*, 2011).

The relationship between the vasculature and the GSCs may be mutually beneficial. GSCs can secrete vascular endothelial growth factor that can induce the tumor angiogenesis (Bao *et al.*, 2006b). Interestingly, the GSCs may possess capability to transform into endothelial cells or to pericytes that cover the vessel walls (Cheng *et al.*, 2013; Ricci-Vitiani *et al.*, 2010). Furthermore, the GBM tumors exhibit vasculogenic mimicry, a process in which tumor cells form tubular structures that resemble blood vessels. The GSCs may form these tubes and enhance the tumor blood flow (Chiao *et al.*, 2011; El Hallani *et al.*, 2010; 2010). However, this phenomena has been challenged by suggesting that rather than transforming into endothelial cells the GSCs are capable of fusing with the existing endothelial cells and forming hybrid vessels (El Hallani *et al.*, 2014). This aids the growth of the tumor vasculature which is responsible for providing the oxygen and nutrient supply to the tumor and thus promotes the tumor growth.

2.4. Controversies in the glioblastoma stem cell hypothesis

In a majority of the investigations conducted so far the identification of the GSCs is based on marker proteins that the cells express. The first markers used for describing the GSC population were CD133 and nestin. They were both promising candidates because they are expressed by the neural stem cells and both of their expression is associated with poor patient survival (Zhang *et al.*, 2008). Furthermore, CD133 proved out to be essential for GSC maintenance since silencing of CD133 reduced the proliferation and self-renewal capability of the GSCs (Brescia *et al.*, 2013). However, the role of CD133 as a universal marker for the GSCs was challenged once it was observed that also CD133-negative cells were able to form tumors in rodents (Beier *et al.*, 2007; Wang *et al.*, 2008). In addition, the CD133-negative cells were able to give rise to CD133-positive cells in rats (Wang *et al.*, 2008). This observation

was partly explained by the finding that the CD133 can shuttle between the plasma membrane and cytoplasm of the cells (Brescia *et al.*, 2013). Majority of the experiments in which the GSCs are pooled based on CD133 utilize fluorescent activated cell sorting based on membranous CD133 expression. Therefore, these studies could miss the GSCs population where CD133 is cytoplasmic at the time of sorting.

After that, several new GSC markers have been searched in hopes of clarifying the characterization of the GSCs. Mostly proteins that are expressed by the normal neural stem cells have been considered as potential markers. The suggested proteins include several transcription factors NANOG, OCT4, SALL4, SOX2, STAT3, Olig2, Bmi1, c-Myc, KLF4 and cell adhesion receptors CD44, L1CAM, integrin alpha6 and CD15 (reviewed in (Bradshaw *et al.*, 2016)). However, none of these markers have been exclusive in describing the GSC population still today. Based on genetic profiling the GBMs consist of both diploid and aneuploid cells which have different tumorigenic properties *in vivo* (Stieber *et al.*, 2014). The aneuploid cells formed tumors more aggressively in mice and resulted in shorter survival times. Since the enhanced tumorigenity is characteristic for GSCs it could be expected that they are mostly aneuploid cells. However, when the GSCs were isolated using different cell surface markers the percentage of the diploid and aneuploid cells within the cell pool varied (Stieber *et al.*, 2014). The ratio of the diploid and aneuploid cells was altered depending on the marker used for the sorting. This suggests that the separation of the GSC population based on cell surface markers cannot discriminate the genetic heterogeneity within the tumor. Therefore, new studies and methods to isolate and characterize the GSCs are needed. Furthermore, it should be considered if stemness could be discriminated by a functional criteria instead of specific markers.

Furthermore, the hierarchical cell division theory of the GSCs has not been proved. Recent report studying the matching pairs of GSCs and their differentiated tumor cell counterparts suggest that most of the GBM cells have the capability to present stem cell characteristics depending on the environment (Schneider *et al.*, 2016). Moreover, both the hypoxic tumor environment and the FGF secreted by adjacent endothelial cells were capable of reprogramming the differentiated tumor cells into more stem like state (Fessler *et al.*, 2015). Therefore, it still remains unsolved whether all the GBM cells possess stem-like cell properties or whether the GSCs are a separate pool of cells within the tumor.

3. Netrins

Netrins constitute a conserved family of extracellular matrix proteins. They have been named after a Sanskrit word netr which means the one who guides. Five secreted netrins, (NTN1-5) and two membrane bound forms (NTN-G1 and NTN-G2) have been discovered in vertebrates (Nakashiba *et al.*, 2002; Serafini *et al.*, 1996; Serafini *et al.*, 1994; Yamagishi *et al.*, 2015; Yin *et al.*, 2000). NTNs 2 and -3 are homologous to each other. NTN2 is expressed in avians and zebrafish while NTN3 is the counterpart in mammals. Uncoordinated-6 (UNC6) and netrin-A and -B (NetA and –B) represent the *Caernohabtidis elegans* (*C. elegans*) and *Drosophila melanogaster* (*D. melanogaster*) counterparts of the vertebrate NTNs (Harris *et al.*, 1996; Mitchell *et al.*, 1996a). NTN1 is the prototype of the family and is most widely studied.

Structurally NTNs resemble laminins and consist of three distinct domains: N-terminal, central and C-terminal domains (figure 3). The N-terminal domain of NTNs 1-3 and NTN-G1-2 is homologous to laminin gamma chain whereas in NTN4 it resembles laminin beta chain

(figure 3). The central part of NTNs contains three EGF repeats homologous to laminin domain VI (Serafini *et al.*, 1994). On the contrary, the C-terminal domain, called NTR domain, is not related to the laminin domains. This domain shares similarity with the C-termini of complement proteins, Frizzled-related proteins, type I collagen C-proteinase enhancer proteins, and tissue inhibitors of metalloproteinases (Banyai and Patthy, 1999). Additionally, the membrane bound NTN-Gs have glycosylphosphatidylinositol anchor in their C-terminal tail (Nakashiba *et al.*, 2002). Furthermore, the newly found member of the family, NTN5 lacks the N-terminal domain and one of the EGF repeats (Yamagishi *et al.*, 2015).

Within this thesis the functions of NTN1 and -4 in glioblastoma were investigated. Therefore their basic functions during development and cancer are reviewed in the following chapters.
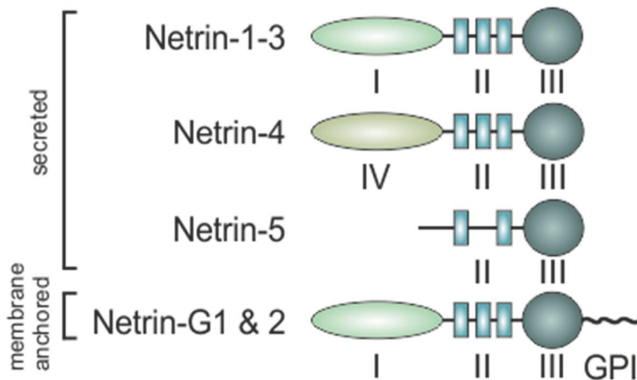
Figure 3. Schematic illustration of the structure of netrins. I: N-terminal domain related to laminin γ-chain, II: 3 EGF repeats, III: NTR domain, IV: N-terminal domain related to laminin β-chain, ~: GPI-anchor

3.1 Various roles of Netrins in development

3.1.1. Netrin-1 in axon guidance

NTNs were first investigated in *C. elegans* where the loss of UNC6 resulted in defects in axon pathfinding (Brenner, 1974; Hedgecock *et al.*, 1990). Subsequently, similar defects were discovered in vertebrates upon partial NTN1 deletion (Serafini *et al.*, 1996). Recently, these findings were confirmed utilizing a mouse model exhibiting complete loss of NTN1. It was found to be embryonically lethal approximately by embryonic day 14.5 due to the failed axon pathfinding (Bin *et al.*, 2015).

NTN1 plays a dual function in axon guidance: it can either act as axon attracting or repelling cue (Hong *et al.*, 1999). These effects are mediated via two receptor families: the deleted in colorectal cancer (DCC) family that contains DCC and neogenin receptors (Engelkamp, 2002; Keino-Masu *et al.*, 1996) and the uncoordinated-5 (UNC5) family comprised of UNC5A-D (Leonardo *et al.*, 1997). The axon attracting effects appear to be mediated via the DCC receptors in coordination with the Down syndrome cell adhesion molecule (DSCAM) and repellent effects via binding to the UNC5 receptors (Keino-Masu *et al.*, 1996; Leonardo *et al.*, 1997; Ly *et al.*, 2008).

The axons sense their environment via specialized structures called growth cones, which are very motile and responsive to many extracellular guidance signals (Geraldo and Gordon-Weeks, 2009; Kolodkin and Tessier-Lavigne, 2011; Lowery and Van Vactor, 2009). The shape of the growth cones resembles human hand: it contains spike like filopodial protrusions and sheet like lamellipodia between the spikes. The growth cones undergo fast changes during the axon guidance. These structural changes are dependent on the microtubule and actin dynamics within the growth cone (Geraldo and Gordon-Weeks, 2009). To be able to turn the growth cone must be able to grow from the other side while retracting from another. Because the axon extensions are very far away from the cell body and nucleus, it is essential that the proteins needed for the growth of the axon can be produced locally (Piper *et al.*, 2015). In this process the microtubules are needed for polypeptide chain elongation and for the delivery of ribonucleoparticles whereas the F-actin is needed for the initiation of the protein synthesis.

These growth cones are responsive to a diffusive NTN gradient secreted by the floor plate cells in the developing embryo. NTN1 acts as a polarizing agent which leads to DCC and DSCAM clustering and to the coupling of microtubules on one side of the growth cone (Huang *et al.*, 2015; Jain and Welshhans, 2015; Qu *et al.*, 2013; 2013). These receptors then function as signaling platforms, which can regulate both the local protein synthesis and microtubule growth to orient the axon steering (Piper *et al.*, 2015; Tcherkezian *et al.*, 2010). When the axon crosses the midline it starts to express UNC5 receptor and its response to NTN1 changes to repulsive (Leonardo *et al.*, 1997). This allows the elongation of the axon on the other side of the embryo.

3.1.2. Functions of netrins in the morphogenesis of epithelial structures

In addition to axon guidance, NTNs have been suggested to play a role in several developmental processes of various epithelial structures. During lung development NTN1- and -4 are expressed by the epithelial cells on the stalk area of the developing lung bud (Dalvin *et al.*, 2003; Liu *et al.*, 2004). After secretion NTNs accumulate to the basement membrane or to the epithelial cells located behind the tip cells (Liu *et al.*, 2004). UNC5B receptor has opposite expression pattern than NTNs and is expressed by the tip cells of the budding lung. In addition, the tip cells show increased phosphorylation of ERK-proteins. Interestingly, the addition of exogenous NTN to 3D-lung bud cultures abolish the budding or even reverse its direction towards the lumen of the lung bud (Liu *et al.*, 2004). It also decreases the overall levels of phosphorylated ERK. This suggests that NTNs at the stalk area prevent the lung bud formation by reducing ERK phosphorylation. Authors examined possible receptors involved and suggest UNC5B to mediate this cascade but do not exclusively rule out integrins or other NTN receptors.

During mammary gland development NTN1 is expressed at preluminal cells of terminal end buds (Srinivasan *et al.*, 2003). Neogenin on the other hand is expressed by cap cells neighboring the preluminal cells. During this process mammary gland branches elongate via growth of the cap cells on the terminal end buds. It is important that the terminal end bud keeps its correct organization where the cap cells are in contact with the luminal cells behind them (Silberstein, 2001). In mammary glands where both NTN1 and neogenin are deleted, the cap cells move uncoordinatedly and creat gaps between the luminal cells and the cap cells (Srinivasan *et al.*, 2003). In addition, mammary glands lacking NTN1 or neogenin display loss of adhesion between the cap and luminal cells although their expression of E- and P-

cadherin remain normal. Therefore NTN1-neogenin interaction may be important stabilizer of the epithelial cell adhesions.

In developing pancreas NTN1 is expressed in various populations of epithelial cells in ductal structures, developing islet cells and around undifferentiated acinar structures (Yebra *et al.*, 2003) whereas NTN4 is expressed in blood vessels within ductal structures (Yebra *et al.*, 2011). However, both NTN1 and NTN4 serve as adhesive surface to pancreatic epithelial cells (Yebra *et al.*, 2003; Yebra *et al.*, 2011). NTN1 and alpha6beta4 and alpha3beta1 integrin receptors co-localize on the surface of the epithelial cells. Furthermore, NTN1 binding to integrins via its C-terminal domain mediate the migratory effects (Yebra *et al.*, 2003). The two integrin receptors co-operate in binding to NTN1 and possibly activate c-MET receptor and subsequent epithelial cell migration pathways. NTN1 induces the migration in a haptotactic manner only and not chemotactically as during axon guidance. Interestingly, majority of the cells migrating along NTN1 are undifferentiated pancreatic progenitor cells. Similarly, NTN4 is observed to bind to integrins alpha2beta1 and alpha3beta1 (Yebra *et al.*, 2011). This binding induces the expression of several cell differentiation related genes suggesting that NTN4 is controlling the differentiation of pancreatic epithelial cells. (Yebra *et al.*, 2011).

During invertebrate development, NTN acts as polarizing factor. Migration of specialized gonadal cell, anchor cell, of *C. elegans* is dependent on the guidance of UNC-6 and UNC-40, *C. elegans* counterparts for NTNs and DCC, respectively (Hagedorn *et al.*, 2013; Sherwood and Sternberg, 2003; Ziel *et al.*, 2009). UNC-6 clusters to the basement membrane through which the anchor cell invades to reach the vulval epithelium and creates vulval-uterine connection. Contact of anchor cell with UNC-6 leads to rapid polarization of the anchor cell which then allows it to invade through the basement membrane cells (Wang *et al.*, 2014). During the polarization UNC-40 receptors cluster to the site of UNC-6 source, followed by the modulation of the F-actin cytoskeleton. This clustering marks the place for the invasive protrusion.

During the development of *D. melanogaster* NTNs are involved in the development of the midgut. First, two masses of mesenchymal primary midgut epithelial cells (PMEC) are located in the opposite ends of the embryo (Tepass and Hartenstein, 1994). They start to migrate towards each other along the visceral mesoderm cells that reside on the outer membranes of the embryo (Tepass and Hartenstein, 1994). In the course of the migration the PMECs undergo mesenchymal to epithelial transition and form epithelium (Tepass and Hartenstein, 1994). The visceral mesoderm cells secrete NetA and -B (*D. melanogaster* counterparts for NTNs) while the PMECs express NTN receptor Frazzled (*D. melanogaster* counterpart for DCC) (Mitchell *et al.*, 1996b). NetA and –B function parallel on this process and are responsible for maintaining the motility and correct shape of the migrating PMECs. Moreover, they induce the polarization and subsequent mesenchymal to epithelial transition of the PMECS. This is mediated via the clustering of Filamin-1, F-Actin and E-cadherin to the contact points with the visceral mesoderm (Pert *et al.*, 2015).

3.2. Netrins in cancer

3.2.1. Netrin-1 as a regulator of cancer cell invasion
NTN1 has been linked to the motility of numerous cancers (table 1). These include both epithelial cancers including colorectal, breast, pancreatic and hepatocellular cancers. In

addition, NTN1 has been linked to the progression of nervous system cancers such as medulloblastoma and neuroblastoma. NTN1 has various effects to cell behavior and will be discussed in more detail in following sections.

Table 1. Netrin-1 expression and functions in various cancers. + expression has no change in comparison to normal tissue, ++ modest increase, +++ strong upregulation and − indicates decreased expression level (modified from (Ylivinkka *et al.*, 2016))

Cancer type		Colon	Breast	Pancreatic	Lung	Liver	Medullo-blastoma	Neuro-blastoma
Expression in tumor tissue	low grade/ non-metastatic tumors	N/R	+	-	+++	+++	N/R	N/R
	high grade/ metastatic tumors	+++	+++	+++	+++	+++	+++	+++
	brain metastases	+	++	N/R	+++	N/R	N/A	N/A
Effects	protection from apoptosis	x	x	x	x	N/R	x	x
	motility promoting effect	x	x	x	x	x	x	x
	suggested as biomarker	N/R	N/R	x	N/R	N/R	x	x

3.2.1.1. Epithelial cancers

In a majority of colorectal cancers both DCC and UNC5 receptors are downregulated (reviewed in Mehlen and Tauszig-Delamasure, 2014). However, the majority of tumors are expressing NTN1 (Paradisi *et al.*, 2008). In addition, colon carcinoma and adenoma cell lines show upregulated expression of NTN1 whereas the expression of DCC or UNC5 receptors were downregulated (Paradisi *et al.*, 2008; Rodrigues *et al.*, 2007). NTN1 upregulation promotes invasiveness and growth of the cancer cells lacking DCC both in vitro and in vivo (Rodrigues *et al.*, 2007). The invasion promoting effects of NTN1 were mediated via RhoA, Rac1, Cdc42 and PI3K signaling. On the contrary, DCC acts as a tumor suppressor gene by reducing cancer cell invasion and metastasis *in vivo* (Rodrigues *et al.*, 2007). DCC expressing tumor xenografts hava same growth potential as control tumors but invade significantly less to lymph nodes or to lungs of the mice (Rodrigues *et al.*, 2007). Furthermore, under hypoxic conditions DCC expression increas the percentage of apoptotic cells but not under normoxic conditions. Interestingly, NTN1 upregulation can not rescue DCC induced apoptosis (Rodrigues *et al.*, 2007) supporting the pro-invasive rather than survival promoting function of NTN1.

In breast cancer NTN1 is expressed especially in the metastatic cell lines (Fitamant *et al.*, 2008). When NTN1 expression was analyzed in human tumor biopsies it was strongly expressed in tumors with lymph node or other distant organ metastasis while tumors that

were restricted to breast area displayed only modest expression (Fitamant *et al.*, 2008). Furthermore, NTN1 expression was analyzed in two mouse breast cancer cell lines, 67NR and 4T1, which differ in their metastatic capability. Interestingly, the non-metastasizing 67NR cell line has lower NTN1 expression than metastatic 4T1 cell line. Expression pattern of classical NTN receptors does not differ between these cell lines. These finding suggest a pro-invasive role for NTN1 in breast cancer but the cellular mechanisms remain largely unknown.

Moreover, NTN1 expression has been found to increase pancreatic ductal adenocarcinoma (PDAC) cell invasiveness and apoptotic resistance. In addition, it serves as adhesive surface for the cells *in vitro* (Dumartin *et al.*, 2010). NTN1 was also observed to mediate the neural invasion of PDAC in a mouse model (Wang *et al.*, 2015). The expression of NTN1 is regulated via NFkb signaling activation (Wang *et al.*, 2015) similarly as observed previously during colorectal cancer progression (Paradisi *et al.*, 2008; Paradisi *et al.*, 2009). In addition, NTN1 also serves as prognostic factor in pancreatic cancer (Link *et al.*, 2007). On the contrary, a recent study suggest NTN1 to be a tumor suppressor in low-grade pancreatic adenocarcinoma via suppressing growth by UNC5B mediated integrin beta4 downregulation (An *et al.*, 2016). However, NTN1 upregulation is observed in higher grade pancreatic adenocarcinomas correspondingly to previous studies (An *et al.*, 2016).

Moreover, NTN1 increases the proliferation and migration of hepatocellular carcinoma cells (Qi *et al.*, 2015). During NTN1 treatment the cancer cells upregulate yes associated kinase (YAP), an oncogene that belongs to the Hippo signaling pathway (Qi *et al.*, 2015). Furthermore, under hypoxic conditions NTN1 induced epithelial to mesenchymal transition and upregulation of invasion promoting inflammatory molecules of the hepatocellular cancer cells (Han *et al.*, 2015; Yan *et al.*, 2014).

3.2.1.2. Cancers of the neural system

NTN1 may promote also the invasiveness of tumors of the nervous system. Its expression increases the invasiveness of medulloblastoma cells via neogenin and UN5B receptors (Akino *et al.*, 2014). Increased levels of NTN1 have been detected in the urine of medulloblastoma patients. Surgical resection of the tumor significantly decreases the levels of NTN1 in the urine suggesting a potential role as a biomarker for NTN1 (Akino *et al.*, 2014). Interestingly, in subcutaneous medulloblastoma mouse xenograft model NTN1 upregulation also increases the vascularity of the tumors (Akino *et al.*, 2014). These findings indicate a role for NTN1 in the progression of tumors in the nervous system.

In an extracranial neural tumor, neuroblastoma, the expression of NTN1 in tumor tissue is associated with poor patient prognosis (Delloye-Bourgeois *et al.*, 2009b). NTN1 is strongly expressed in aggressive, stage 4 neuroblastoma. On the contrary, expression of the DCC receptor is low and UNC5A-B modest in these tumors. NTN1 expression is connected to increased apoptosis resistance of the neuroblastoma cells (Delloye-Bourgeois *et al.*, 2009b). Interestingly, NTN1 inhibition also leads to a decrease in the migratory capacity of the neuroblastoma cells. In a mouse model where NTN1 negative primary neuroblastoma tumors were xenografted into mice the emerging metastatic lesions in lungs or heart are NTN1 positive. This raises the question: why is NTN1 upregulated during the metastatic cascade? Does NTN give advantage in this process? Authors suggest that NTN1 expression allows the tumor cells to survive better outside the primary tumor (Delloye-Bourgeois *et al.*, 2009b). However, considering the evidence of the proinvasive nature of NTN1 in epithelial

cancers it may regulate the invasiveness of neuroblastoma as well. Moreover, a recent study suggests that NTN1 is an independent prognostic factor in brain metastases of various cancers including non-small cell lung cancer, breast cancer and melanoma (Harter *et al.*, 2014).

In the light of these observations NTN1 seems to be strongly linked to cell invasion and metastatic growth. However, not much is known about NTN1 driven molecular mechanisms in cancer cell invasion. Since in many cancers the expression of NTN1 and DCC or UNC5 does not correlate there may be novel signaling pathways mediating the effects of NTN1.

3.2.2. Netrin-4 has a dual function in tumorigenesis

Besides NTN1, also NTN4 is connected to cancer cell invasion. However, its role is contradictory: some reports indicate that NTN4 is a negative regulator of tumor cell motility while others report the opposite.

In breast cancer NTN4 is downregulated in majority of primary tumors while upregulated in invasive tumor cells (Esseghir *et al.*, 2007). Similarly, NTN4 is upregulated in breast and Müllerian carcinoma effusions (Yuan *et al.*, 2011). NTN4 inhibition by microRNA miR-196a results in lowered proliferation and migration rates of cervical cancer cells (Zhang *et al.*, 2013). Upregulation of NTN4 expression is also observed in invasive glioblastoma cells when compared to tumor core (Hoelzinger *et al.*, 2005). Furthermore, NTN4 upregulation in invasive melanoma tumors was recently reported (Jayachandran *et al.*, 2016). Silencing of NTN4 decreases melanoma cell invasiveness *in vitro* and *in vivo.* In gastric cancer NTN4 mediates the proliferation and motility of cancer cells *in vitro* via neogenin receptor (Lv *et al.*, 2015). NTN4 expression in human gastric cancer biopsies is also associated with poor patient prognosis (Lv *et al.*, 2015). This observation is in discordance with previous study where the upregulated NTN4 expression in breast cancer biopsies predicts patient prognosis (Esseghir *et al.*, 2007).

On the contrary, NTN4 protein expression has been also linked to tumor suppressor functions. When screened in a variety of cell-lines NTN4 expression is observed to be higher in normal cells, such as endothelial and epithelial cells and fibroblasts than in transformed cancer cell lines (Nacht *et al.*, 2009). Furthermore, the addition of exogenous NTN4 has little effect on primary cells but inhibited proliferation of cancer cell lines. Interestingly, all cells are responsive to low concentrations of recombinant NTN4 but increasing concentrations show inhibitory effects. In addition, tumor growth suppressing functions of NTN4 have been observed in colorectal cancer *in vivo* (Eveno *et al.*, 2011; Eveno *et al.*, 2013). Upon NTN4 overexpression in colorectal carcinoma cells growth of the primary tumor is suppressed in mouse xenograft model (Eveno *et al.*, 2011; Eveno *et al.*, 2013). Moreover, the number of liver, lung and lymph node metastases is decreased too (Eveno *et al.*, 2013). NTN4 expressing tumors show less tumor angiogenesis and more apoptosis among the tumor cells (Eveno *et al.*, 2011). However, exogenous NTN4 addition does not affect cancer cell apoptosis or proliferation *in vitro*. Therefore, authors suggest that the effect on the primary tumor is due to the reduced tumor angiogenesis.

3.2.3. Netrins as tumor cell survival factors

NTNs have been implicated to act as survival factors in many forms of cancer. Based on these observations Mehlen and colleagues have presented dependence receptor hypothesis where the proapototic functions of the NTN1 receptors are prevented by interaction with NTN1 (Llambi *et al.*, 2001; Mehlen *et al.*, 1998; Mehlen and Bredesen, 2004). Both DCC and UNC5 receptors contain so called death domain in their cytoplasmic tail. When the receptor is not engaged with NTN1 the death domain is exposed and allows the binding of caspase-9. This leads to caspase-3 activation and subsequent apoptosis. However, the caspase activation sequence is prevented during the binding of NTN1 which leads to apoptosis resistance. Therefore, NTN1 upregulation has been suggested to be a way to gain survival advantage for several cancers including colorectal cancer, non-small cell lung cancer, neuroblastoma and metastatic breast cancer (Delloye-Bourgeois *et al.*, 2009a; Delloye-Bourgeois *et al.*, 2009b; Fitamant *et al.*, 2008; Mazelin *et al.*, 2004).

4. Notch signaling

In this thesis NTN1 was observed to interact with various components of Notch signaling pathway. Furthermore, it was detected to induce Notch signaling. Therefore, the basics of Notch signaling and its functions in GBM are discussed in the following chapter.

4.1. Basics of Notch signaling

Notch receptors constitute a conserved family of transmembrane receptors. The name originates from the phenotype of a mutant *D. melanogaster* that had notched wings. Four receptors, Notch1-4 (Ellisen *et al.*, 1991; Lardelli *et al.*, 1994; Uyttendaele *et al.*, 1996; Weinmaster *et al.*, 1992), and five known ligands, Jagged1-2 and delta-like 1,-2 and 4 exist in mammalians. The Notch proteins consists of a large extracellular domain containing 36 epidermal growth factor–like repeats, a single transmembrane domain, and an intracellular domain containing a recombining binding protein suppressor of hairless (RBP-JK) -associated molecule region, ankyrin domains, and a proline-glutamate-serine-threonine-rich region.

The Notch receptors are transcribed in the endoplasmic reticulum as pre-receptors (figure 4.). To yield active receptor they first undergo O-fucosylation in the endoplasmic reticulum and furin cleavage in the S1 site at the Golgi apparatus (reviewed in (Haines and Irvine, 2003)). This is then followed by second glycosylation and transport to cell surface. Once the receptor on the cell surface comes in contact with its ligand on neighboring, signal sending cells it undergoes conformational change and a cleavage by ADAM10 or -17 proteinase (Schroeter *et al.*, 1998). The endocytosis of the ligand to the signal sending cell provides a pulling force which then allows the intramembrane cleavage of the Notch receptor by presenilin1 protease within the γ-secretase complex (Meloty-Kapella *et al.*, 2012; Struhl and Greenwald, 1999). The intracellular part of the receptor is endocytosed (Vaccari *et al.*, 2008) and transported to the nucleus (Schroeter *et al.*, 1998). In the nucleus it binds to mastermind-like 1 (MAML) and RBP-JK to form complex that activates transcription in the target genes (Nam *et al.*, 2006; Wilson and Kovall, 2006).

During developmental processes the Notch signaling pathway is one of the major regulators of cellular differentiation. The tissues that are dependent on Notch include CNS, hematopoietic system, vascular system, muscles, bones and several organs including heart,

skin, kidneys, lungs and pancreas (reviewed in Artavanis-Tsakonas *et al.*, 1995; Kopan and Ilagan, 2009; Xu *et al.*, 2012). Furthermore, the Notch signaling has been connected to several pathological conditions including various cancers, fibrosis, Down syndrome, Alzheimer's disease and multiple sclerosis (reviewed in Lobry *et al.*, 2011; Mathieu *et al.*, 2013; Xu *et al.*, 2012) In this thesis, the Notch pathway functions have been studied in GBM and thus it will focus on reviewing the functions of Notch signaling in GBM in more detail.
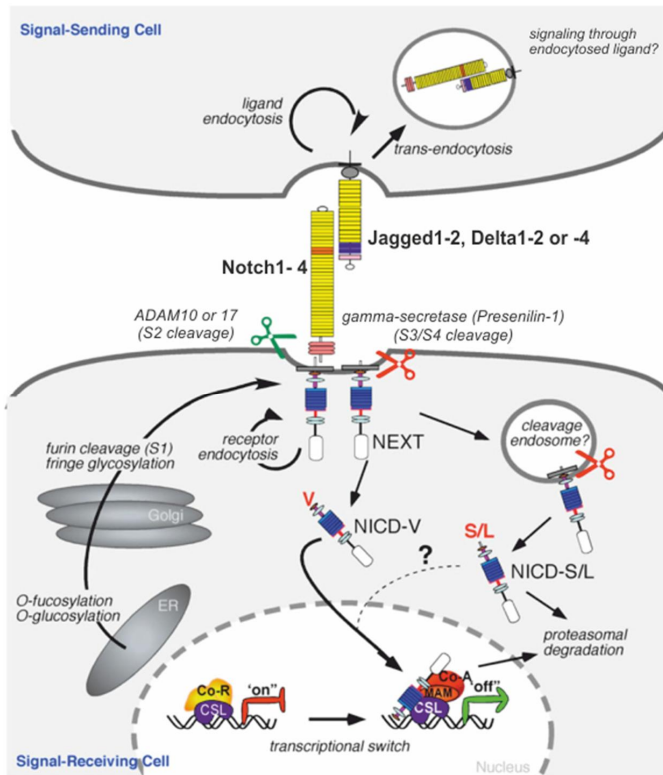
Figure 4. Notch signaling. Schematic illustration of the Notch receptor maturation and activation. Modified from (Kopan and Ilagan, 2009)

4.2. Notch signaling in glioblastoma

The first indications of the importance of Notch signaling in glioma biology were the observations of upregulated expression of Notch1, Jagged1 and Delta-like1 in several long cultured GBM cell-lines and tissues of primary gliomas (Purow *et al.*, 2005; Shih and Holland, 2006). In the glioma tissues strong nuclear Notch staining was detected, which indicated activation of the signaling pathway (Purow *et al.*, 2005). In addition, the conserved Notch-target genes HES1 and HEY1 were expressed in all astrocytomas suggesting activated Notch signaling within the tumors (Chen *et al.*, 2010). Silencing of Notch1, Jagged1 or Delta-like1 with shRNAs in GBM cell-lines decreased their proliferation and tumorigenesis in mice

(Purow *et al.*, 2005). Correspondingly, the inhibition of Notch signaling using γ-secretase inhibitor (GSI) resulted in decreased proliferation of the GBM cell-lines (Chen *et al.*, 2010).

Interestingly, the different Notch receptors may have independent functions in gliomas. Notch1 was observed to be expressed in all astrocytic tumors whereas Notch2 was expressed selectively in a subset of the GBMs (Shih and Holland, 2006). Similarly, another studies noted that Notch2 and Notch4 were highly expressed in GBM whereas Notch1 was more strongly expressed in lower grade astrocytomas (Chen *et al.*, 2010; Dell'albani *et al.*, 2014). It was also suggested that the expression of different Notch receptors could be used as markers for glioma grading (Dell'albani *et al.*, 2014). Furthermore, in a mouse model where activated Notch receptors were overexpressed in the eyes of embryonic mice, Notch3 receptor showed most tumorigenic potential (Pierfelice *et al.*, 2011). The increase in Notch1 or -2 activation resulted in small tumors whereas active-Notch3 overexpression resulted in glioma-like tumors that were invading towards the brain along the optic nerve (Pierfelice *et al.*, 2011).

Notch signaling maintains the neural stem cells in undifferentiated state especially under hypoxic conditions (Gustafsson *et al.*, 2005). Since the GBMs are also very hypoxic, Notch signaling is an interesting candidate pathway for regulating the GSC maintenance. Indeed, the first indications that Notch pathway is linked to the GSCs was made already during the initial discovery of the GSCs. Upon genetic profiling of the newly characterized GSCs, expression of Notch pathway candidates was detected with QRT-PCR analysis (Galli *et al.*, 2004). Later the activation of the Notch signaling in these GSCs was verified by HES1 expression that diminished upon GSI treatment (Fan *et al.*, 2010). The treatment also blocked cell proliferation and reduced the number of nestin- and CD133 positive cells via caspase-3 activation and subsequent apoptosis (Fan *et al.*, 2010). Inhibition of Notch signaling by GSIs also prevented the formation of neurospheres of primary GBM cells suggesting reduced capability to maintain GSCs phenotype (Chen *et al.*, 2010). Furthermore, the GSCs that have high endogenous Notch activation are more sensitive towards the GSI treatment and undergo cell cycle arrest and apoptosis upon the treatment (Kristoffersen *et al.*, 2013). On the contrary, it was reported that Notch inhibition would affect only the initial capability to form the neurospheres but would not affect self-renewal or the clonogenity (Kristoffersen *et al.*, 2013).

Moreover, Notch pathway upregulation and enhanced nestin expression was observed (Shih and Holland, 2006) in spontaneous glioma mouse model induced by constantly active Kras (Holland *et al.*, 2000). It was suggested that Notch signaling could promote the stemness and nestin expression of the GBM cells. This observation was further validated in *in vitro* conditions using U251MG cells. The forced upregulation of the Notch intracellular domain upregulated nestin expression suggesting that nestin may be a transcriptional target of Notch (Shih and Holland, 2006). Furthermore, both local administration of GSI and pretreatment of GSC cell-lines with GSI reduced the capability of GSCs to initiate tumor growth in intracranial xenografts (Chen *et al.*, 2010; Fan *et al.*, 2010). GSI treatment also led to the generation of more differentiated and angiogenic tumors (Kristoffersen *et al.*, 2014).

Since the GSCs are more resistant to radiation and chemotherapy, inhibition of Notch signaling is a putative way to sensitize the cells to treatment. Indeed, the GSI treatment sensitized especially the GSC population to radiation (Wang *et al.*, 2010). Similarly, knockdown of Notch1 or Notch2 increased the radiosensitivity of GSCs whereas the introduction of constitutively active intracellular domains of Notch1 or Notch2 rescued the

GSI induced sensitization (Wang *et al.*, 2010). The inhibition of Notch signaling with GSIs in combination with inhibition of another developmental pathway, Sonic Hedgehog, induced the sensitivity of GSCs to TMZ treatment (Ulasov *et al.*, 2011). Furthermore, the GSI treatment combined with radiation therapy and temozolomide prolonged the survival of orthotopic glioblastoma (Yahyanejad *et al.*, 2016).

In addition to the Notch signaling targeting via GSIs, it has been suggested that targeting the transcription co-activator of Notch signaling would be more effective. Indeed, expression of a dominant negative form of mastermind-like 1 protein caused G0/G1 cell cycle arrest and induced apoptosis in GBM cell lines (Chen *et al.*, 2010). When LN428 cell expressing this dominant negative form of MAML protein were intracranially xenografted to mice the resulting tumors had less Hes1 and Hes5 expression than control tumors suggesting reduced Notch signaling (Chen *et al.*, 2010). Furthermore, the GCSs express the RBPJ transcription activator and require its expression for proliferation and self-renewal (Xie *et al.*, 2016). In addition, silencing of RBPJ in GSCs reduced their tumorigenic properties *in vivo* (Xie *et al.*, 2016).

Encouraged by these preclinical findings the GSIs have been also tested in clinical trials for glioma treatment. To first clinical study patients with various advanced solid tumors were recruited (Krop *et al.*, 2012). 42 glioma patients were also included to the study. Varying dosages (450mg-4200mg) of GSI were orally administered to patients. Also the frequency and length of treatment varied. The most beneficial treatment responses were observed among the glioma patients: one patient with an anaplastic astrocytoma had a complete recovery and the survival time of 10 other glioma patients prolonged (Krop *et al.*, 2012). However, the patient cohort was too small to statistically evaluate the effects regarding the survival of the patients. After these promising results another phase 0/I study was performed aiming to investigate the ability of a GSI based drug to penetrate the blood brain barrier and its effect to Notch signaling and to GSCs within GBM tumors (Xu *et al.*, 2016). The evaluation of the survival of the patients was not the primary objective and only 20 newly diagnosed GBM patients were recruited to this study. GSI was administered as the only drug before surgery and together with normal TMZ dosage and radiation therapy after surgical resection of the tumor. In the study low toxicity was observed as was expected (Xu *et al.*, 2016). The compound penetrated the blood brain barrier well and successfully reduced the expression of Notch signaling pathway components and target genes within the GBM tissue (Xu *et al.*, 2016). However, the concentration within the tumor tissue varied between patients.

Based on these findings targeting Notch signaling in human gliomas is an interesting therapeutic target. More studies should be conducted to evaluate its clinical benefits. The recent advances in the molecular subtyping of gliomas suggest that especially the patients with classical GBM subtypes could benefit from the Notch inhibition therapy, as the tumors within the classical subtype were characterized with highly expressed nestin, Notch pathway components and Sonic Hedgehog signaling pathways (Verhaak *et al.*, 2010). This should be taken into consideration when planning new clinical trials.

AIMS OF THE STUDY

Since NTNs are essential regulators of nervous system development, we hypothesized that they might be important in human gliomas too. This was further supported by the findings that NTN1 regulates several other cancers. At the time I started this research work nothing was known about the roles of NTNs in human gliomas or glioblastomas. Therefore the major aim was to identify receptors and signaling pathways of NTN1 and -4 in glioblastoma cells. In the course of the studies my main interest shifted towards NTN1 and its involvement in the regulation of GBM motility and stemness. This was initiated with the notion that NTN1 was overexpressed in human GBM tumors and in invasive GBM cell-lines.

The specific aims of this work were:

1. To characterize the effects of NTN1 and NTN4 on glioblastoma cells (I, II, III)
2. To identify proteins interacting with and pathways mediating the effects of NTN1 and NTN4 (I, II)
3. To investigate the possible link between NTN1 and GBM invasiveness using *in vitro* and *in vivo* models (II, III)
4. To explore how NTN1 is expressed in human gliomas, does the expression correlate with patient prognosis and where it is localized in human glioma tissues (III)
5. To analyze if NTN1 is associated with the stemness of the GBM cells (III)
6. To decipher the mechanisms how engineered peptide, NTN1(II)FH, inhibits NTN1 signaling in GBM cells (II, III)

MATERIALS AND METHODS

1. Cell lines

The following cell lines were utilized. The articles in which the cell lines were used are referred to with their roman numerals. Astrocytes, U87MG and 293FT cells were cultured according to manufacturer's instructions. U251MG and U373MG cell lines were cultured in Dulbecco's modified essential culture medium (DMEM) supplied with 10 % heat inactivated fetal calf serum (Gibco, USA), 100 IU/ml penicillin, 50 mg/ml streptomycin and 1% L-glutamine. GBM9 and GBM10 were isolated from fresh surgical GBM biopsies (see protocol below) and cultured in serum free conditions in DMEM/F12 supplemented with 100 IU/ml penicillin, 50 mg/ml streptomycin, B27 supplement, EGF 20ng/ml, FGF 20ng/ml.

Cell line	Origin	Source	Article used in
Astrocytes	human brain	Lonza	I, II
GBM10	human GBM	established by us	III
GBM9	human GBM	established by us	III
U251MG	human GBM	Health Sciences Resource Bank, Japan	I, II, III
U373MG	human GBM	DR. Bengt Westermark Uppsala	I, II, III
U87MG	human GBM	ATCC	I, II, III
293FT	human embryonal kidney	Invitrogen	I, II, III

2. Reagents

Name	Purpose	Manufacturer	Articles used in
rNTN1	recombinant human NTN1	R&D Systems	III
rNTN4	recombinant human NTN4	R&D Systems	I
DAPT	Notch inhibitor	Sigma Aldrich	II
Fugene	Transfection reagent	Promega	I, II
Turbofect	Transfection reagent	Thermo Fisher Scientific	I, II, III
Lipofectamine	Transfection reagent	Invitrogen	II
rEGF	recombinant human EGF	R&D Systems	III
rFGF	recombinant human basic FGF	R&D Systems	III
DABCO	antifading reagent	Sigma Aldrich	I, II, III
Matrigel	Matrix used for 2D and 3D invasion assays	BD Biosciences and Corning	I, III
Biotin	used for biotinylation of all cell surface proteins	Pierce Biotechnology	I
D-luciferase	substrate for bioluminescence imaging	Regis Technology	III

3. Antibodies

The following antibodies were used. The antibodies target human proteins unless stated otherwise.

Name	Source	Manufacturer	Articles used in
beta-tubulin	rabbit	Santa Cruz Biotechnology	I, II
BrdU	mouse	Santa Cruz Biotechnology	I
CD133	mouse	Miltenyi Biotech	III
Clathrin	mouse	Thermo Scientific	II
GAPDH	mouse	Sigma Aldrich	II
HA-11	mouse	Covance	I, II, III
HA-tag	rabbit	Cell Signaling	III
HA-tag	rat	Roche	III
ITGB4	chicken	Sigma-Aldrich	I
Jagged1 (C-20)	goat	Santa Cruz Biotechnology	II, III
lamin A/C	rabbit	Novus Bio	III
mouse CD31	rat	BD Pharmingen	III
Nestin	rabbit	Millipore	III
Notc2 ICD	rabbit	Millipore	II, III
Notch2 (25–255)	rabbit	Santa Cruz Biotechnology	II, III
Notch2 cleaved (D1733)	rabbit	Immunoway	III
NTN1	mouse	Enzo Biosciences	II
NTN1	chicken	Neuromics	III
NTN4	goat	R&D Systems	I
Phalloidin	*Amanita phalloides*	Sigma Aldrich	II
phospho ERK 1/2	rabbit	Cell Signaling	I
phospho mTOR	rabbit	Cell Signaling	I
phospho-AKT	rabbit	Cell Signaling	I

4. Vectors and expression constructs

Full-length NTN1 and NTN4 were cloned into pLVX-Puro expression vector. In addition, three fragments of NTN1 and five fragments of NTN4 were cloned into pLVX-Puro vector. Since the fragments were lacking the signal sequence of full-length proteins, CD33 signal sequence was inserted into the 5' end of the coding region of each construct. In addition, HA and Flag tags were inserted into the C-terminal end of the proteins. All constructs are presented as a table below.

Name	Protein coded	Tags	Plasmid	Reference
NTN1	Full-length NTN1	none	pLVX-Puro	II, III
NTN1FH	Full-length NTN1	Flag and HA tagged	pLVX-Puro	II, III
NTN(I)FH	Amino acids 1-283 of NTN1	Flag and HA tagged	pLVX-Puro	II, III
NTN1(II)FH	Amino acids 282-486 of NTN1	Flag and HA tagged	pLVX-Puro	II, III
NTN1(III)FH	Amino acids 485-604 of NTN1	Flag and HA tagged	pLVX-Puro	II, III
NTN4	Full-length NTN4	none	pLVX-Puro / pCDNA3	I
NTN4HF	Full-length NTN4	Flag and HA tagged	pLVX-Puro / pCDNA3	I
Fragment 1	Amino acid 1-261 of NTN4	Flag and HA tagged	pLVX-Puro / pCDNA3	I
Fragment 2	Amino acid 261-445 of NTN4	Flag and HA tagged	pLVX-Puro / pCDNA3	I
Fragment 3	Amino acid 261-516 of NTN4	Flag and HA tagged	pLVX-Puro / pCDNA3	I
Fragment 4	Amino acid 445-628 of NTN4	Flag and HA tagged	pLVX-Puro / pCDNA3	I
Fragment 5	Amino acid 516-628 of NTN4	Flag and HA tagged	pLVX-Puro / pCDNA3	I

5. Lentivirus mediated manipulation of gene expression (I, II, III)

Lentiviruses were produced in 293FT cells. The producer cells were transfected with lentiviral packaging and envelope plasmids (pCMVdr8.74 and pMD2-VSVG; Addgene, Cambridge, MA) together with the intended expression construct. 48 hours later the virus containing supernatants were collected and virus titer determined. The target cells were then transduced with the lentivirus supernatant and infected for 24 hours. For stable expression, cells were selected with culture medium containing 5µg/ml puromycine. The expression of selected gene was also ensured with QPCR and immunofluorescence.

For the overexpression of indicated genes an empty pLVX-Puro was used as infection control. In the case of gene silencing, five shRNAs targeting the silenced genes were tested. The shRNAs were obtained from RNAi Consortium through Open Biosystems. A nontargeting scrambled shRNA construct was used as infection control.

6. Total RNA extraction, reverse transcription, and real-time quantitative-PCR (I, II, III)

Total cellular RNA was purified using NucleoSpin® RNA II kit (Macherey-Nagel). Reverse transcription was carried out using iScript cDNA Synthesis Kit (Bio-Rad). The relative amount of mRNA of the indicated genes was measured with quantitative real time PCR using TaqMan

Assays-on-Demand gene expression products (Applied Biosystems, Foster City, CA), and iQ Supermix (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for each sample. PCR was run using CFX96 machinery (Bio-Rad) and the relative expression levels were quantified with ΔΔCT method with Bio-Rad CFX Manager software.

7. *In vitro* cell proliferation assays (I, III)

Cell proliferation was measured with bromodeoxyuridine assay (I) and EdU assay (II). In bromodeoxyuridine assay cells were cultured on a 96-well plate (PerkinElmer, Waltham,MA). Subsequently, the medium was replaced to medium containing 5-bromo-2-deoxyuridine (BrdU) and incubated for 70 (serum containing) or 120 minutes (serum starvation). After incubation, the BrdU labeling medium was removed, and the cells were washed with phosphate-buffered saline (PBS) twice. The cells were then fixed with 70% ethanol containing glycine at −20°C for 30 minutes. After washing with PBS, the cells were incubated in 2 M HCl at room temperature for 60 minutes. Subsequently, the cells were rinsed and treated with PBS containing 3% bovine serum albumin (BSA) at room temperature for 30 minutes and incubated with anti-BrdU antibodies. The primary antibody was detected by A594 Alexa Fluor secondary antibodies (Invitrogen). After Hoechst staining at 4°C for 15 minutes, cells were washed again. Finally, the images were captured and quantified by using ArrayScan 4.5 high-content-screening system (Cellomics, Pittsburg, PA).

In the EdU assay the Click-IT EdU Imaging Kit (Life Technologies) and the standard protocol of the manufacturer was used. In short, U87MG or U373MG cells were treated with 10 µM of EdU for 60 min prior to fixation. Cells were stained using Click-IT EdU Imaging Kit with AlexaFluor-594 conjugate (Thermo Scientic). The nuclei were visualized with 5 µg/ml of Hoechst 44432 (Invitrogen). The proliferation rate was determined by dividing the total cell number with EdU labeled cell number using ANIMA software (Rantanen *et al.*, 2014).

8. 2D cell motility assays (I, II)

Two types of 2D motility assays were utilized: scratch induced wound closure assay and Matrigel invasion assay.

In the scratch induced wound closure assay, cells were cultured under normal conditions on a 24-well plate until 90% confluency. A linear wound was scratched into the middle of each well using micropipette tip. Detached cells were removed by washing with PBS. Cell culture medium containing 0,5% serum was added to the well. The migration of the cells was monitored with Axiovert 200 inverted epifluorescence microscope. The migration rate was assessed by analyzing the wound width at 0 and 8 hours timepoints using ImageJ software. For the Matrigel invasion assays cell culture inserts with 8µm pore size (Becton Dickinson) were coated with Matrigel (BD Biosciences) and dried. 20 000 cells were seeded on top of the coated insert in a serum free DMEM. Serum containing DMEM was administered to the lower chamber. Cells were allowed to invade for 7 hours in a cell culture incubator. Next, the cells were fixed with 40% MeOH, 10% CH3COOH and stained with Coomassie Brilliant Blue. Cells that had not migrated through the inserts were removed. The inserts were imaged with Axiovert 200 microscope (Zeiss) and the area covered by invaded cells was measured with Image J software.

9. Cell survival assay (I)

Cells were cultured on glass coverslips overnight followed with 72 hour serum starvation. Next, the coverslips were washed with PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 20 minutes. Apoptotic cells were detected using *In Situ* Cell Death Detection Kit (Roche, Switzerland) according to the manufacturer's instructions. Finally, the coverslips were mounted with VECTASHIELD antifading reagent (Vector Laboratories, Burlingame, CA) which included 4',6-diamidino-2-phenylindole for nuclei staining. The images were captured using the 20x objective of the Axioplan microscope (Carl Zeiss, Inc, Thornwood, NY), acquired with AxioCamHRc camera (Carl Zeiss) and AxioVision3.1 software (Carl Zeiss).

10. Immunofluorescence analysis of cells (I, II)

Cells were grown on class coverslips and indicated treatments were applied. The cells were fixed with 4% PFA and permeabilised with 0,1% Tx100 in TBS. Then cells were incubated with 3% BSA or 5% non-fat milk in PBS to prevent the non-specific binding of antibodies. Primary antibodies were diluted to the blocking buffer and incubated on the cells for 60 minutes. After incubation the excess primary antibodies were removed by washing with PBS. The cells were then incubated 30 min with fluorescent labelled Alexa secondary antibodies (Invitrogen) which were diluted to the blocking buffer. Subsequently, nuclei were visualized with Hoechst 33342 (Sigma Aldrich). Excess antibodies were again washed away with PBS and lastly with water. TH coverslips were mounted on microscopic slides with Mowiol mounting medium (Calbiochem) supplemented with 6mg/ml DABCO (Sigma Aldrich). The cells were analyzed under Zeiss Axioplan 2 or Zeiss AxioImager.Z2 upright epifluorescence microscopes. Depending on the microscope used the images were acquired using Zeiss digital AxioCam grayscale camera and AxioVision 4.6 software or Hamamatsu Orca Flash 4.0 LT, 4 megapixel monochrome sCMOS camera and Zeiss Zen 2 pro software, respectively. Alternatively, Zeiss LSM 510 Meta or LSM 880 laser scanning confocal microscopes were utilized for capturing micrographs.

11. SDS-PAGE and immunoblot analysis (I, II, III)

Cells were harvested by lysing with Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and complete protease inhibitors (Roche). Nuclei were removed by centrifugation. The proteins were electrophoretically separated in 4%-20% gradient Tris-HCl polyacrylamide gels (Lonza and Bio-Rad). The proteins were then transferred onto nitrocellulose membranes (Bio-Rad) using semi-dry method and Trans-Blot Turbo apparatus (Bio-Rad). The efficiency of the transfer was confirmed with Ponceau staining of the membrane. The membrane was then incubated with 3% BSA or 5% non-fat milk and 0,05% Tween-20 in TBS. Primary antibodies were diluted into the blocking buffer, administered to the membrane and incubated at 4°C overnight. Membrane was washed several times. Horseradish peroxidase conjugated secondary antibodies (GE Healthcare) were diluted to the blocking buffer and incubated with the membrane for 60 min at room temperature. Finally, the filter was washed and the immunoreactive proteins were visualized by ECL-reagent (GE Healthcare) and exposing to X-ray film (Fujifilm). The films were developed using Kodak X-OMAT 2000 equipment (Kodak).

Phosphospecific Western blot analysis was performed according to the manufacturer's instructions (Cell Signaling Technology, Danvers, MA).

Quantification of western blots that were processed as described above, was performed with ImageJ software from the scanned X-ray film images. Alternatively a methods based on fluorescently labelled secondary antibodies was utilized. After transferring the proteins to nitrocellulose membranes, immunoblotting was performed according to the protocol suggested by Li-Cor Biosciences. The membrane was scanned with the Odyssey Infrared Imaging System (Li-Cor Biosciences). Intensities of the bands were analyzed using Odyssey 2.1 software (Li-Cor Biosciences).

12. Identification of binding partners of netrins (I, II)

Cells expressing tagged NTN1 fragments were lysed with lysis buffer containing Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and complete protease inhibitors (Roche). The cell lysates were incubated with FLAG–Sepharose on a shaker at +4°C for 12 hours. Next, the Sepharose particles were collected by centrifugation and washed with the lysis buffer. The proteins bound to the FLAG-Sepharose were detached by incubation with 150 ng/µl FLAG peptide (Sigma Aldrich) at +4°C for 2 hours and subsequently incubated with anti-HA Sepharose in an end-over-end rotating shaker for 1 hour. After incubation the Sepharose particles were washed with the lysis buffer. Finally, the Sepharose-bound proteins were eluted by incubating the particles with non-reducing Laemmli sample buffer at 100°C for 5 minutes. The eluted proteins were then separated by SDS-PAGE (10–20% gradient gels) and visualized by Proteo Silver silver staining kit (Sigma Aldrich) according to manufacturer's protocol. For the identification, proteins were in-gel digested with trypsin and the resulting peptides were identified by liquid chromatography–tandem mass spectrometry analysis as previously described (Ohman *et al.*, 2014).

13. Monitoring the Notch signaling activation (II)

Notch signaling activation was monitored using luciferase reporter assays. A Notch response element containing plasmid TP1-luc(981-6) and a Notch2-intracellular-domain-encoding plasmid, pEF-BOSneoSE-mNotch2 RAMIC, were acquired from RIKEN BioResource Center DNA (Kato *et al.*, 1996; Kurooka *et al.*, 1998). pRL-TK Renilla luciferase vector (Promega) was used as a transfection control in luciferase assays.

A plasmid containing the Notch response element controlling the transcription of the luciferase gene (TPI-luc) was co-transfected by Fugene 6 transfection to target cells together with constitutive expression of Renilla luciferase (pRL-TK) and pCR 3.1 empty vector (Invitrogen) (Kurooka *et al.*, 1998). In the positive control pCR 3.1 vector was replaced with the intracellular domain of Notch2 containing pEF-BOSneoSE-mNotch2 RAMIC (Kato et al., 1996). Transfection was done according to manufacturer's instructions. After 48 hours the cell lysates were collected and luciferase activity was measured with the Dual Luciferase Assay Kit (Promega) using a Digene DCR-1 luminometer.

14. Biotinylation of the cell surface proteins (II)

First cells were washed with cold phosphate-buffered saline. EZ-Link Sulfo-NHS-LC-LC-Biotin (0.5 mg/ml) in PBS (Pierce Biotechnology) was administered to cells and allowed to react with the cell surface proteins on ice for 1 hour. Excess biotin was removed by washing the cells with 150 mM glycine in Tris-buffered saline, pH 7.5. Next, the cells were lysed with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate (DOC), 0.1% SDS supplemented with Complete protease inhibitor (Roche). The nuclei were removed from lysates by centrifugation and the cell lysates were preabsorbed with protein-G–Sepharose (GE healthcare) at +4°C for 1 hour. Next, the lysates were then incubated with either anti-Notch2 extracellular or anti-Jagged1 antibodies at +4°C for 1 hour. The antibodies together with their binding proteins were precipitated with protein-G–Sepharose. Subsequently, the Sepharose particles were washed and the bound proteins were eluted with Laemmli sample buffer by boiling at +100°C for 5 minutes. The eluted, biotinylated proteins were analyzed with SDS-PAGE and immunoblotting with horseradish peroxidase conjugated streptavidin. The biotinylated Notch2 or Jagged1 proteins were identified based on their expected molecular weight.

15. Tissue microarray analysis (TMA) (III)

All experiments involving the use of human tumor tissue was authorized by the Operative Division of Ethical Committee of Helsinki University Hospital (license number 276/13/03/02/2013) and The National Supervisory Authority for Welfare and Health (license number 95/06.01.03.01/2014). NTN1 expression was analyzed in two paraffin embedded tissue microarrays: an array containing 103 low grade glioma samples previously described in (Tynninen *et al.*, 2004) and an array consisting of 40 GBMs. 5 μm thick tumor sections were cut on SuperFrost+ slides (Menzel-Gläser), deparaffinized in xylene and rehydrated in a decreasing ethanol gradient series. The sections were incubated in 3% hydrogen peroxide for 30 minutes to quench the endogenous peroxidase activity. Subsequently, the epitope was retrieved using heat induced epitope retrieval in sodium citrate buffer (10 mmol/L, pH 6.0). Next, the sections were incubated with NTN1 overnight in +4 degrees. Excess primary antibody was washed away and secondary antibodies from ImmPress (Vector laboratories) staining kit was added according to manufacturer's instruction. Finally, the NTN1 on tissues was detected with a DAB Peroxidase Substrate Kit (Vector Laboratories) 15 minutes at room temperature and counterstained using hematoxylin. Kidney tissue was used as positive control. The NTN1 expression was evaluated and scored by experienced pathologist. Samples were scored either NTN1 positive or negative.

16. Tissue immunofluorescence analysis (III)

In addition, we collected fresh glioblastoma tumor biopsies directly from the surgery. The tissue was washed with DMEM/F12, embedded into OCT compound (Sakura Biotech) in a cryomold and fresh frozen using liquid nitrogen. After freezing the tissueblock was stored in -80°C. For immunofluorescence staining 7 μm thick sections were cut with Cryotome (Sakura Biotech) and transferred onto microscope slides for staining. The slides were either stained directly or stored in -20°C.

For immunofluorescence staining, 7 µm thick sections were prepared and transferred onto SuperFrost+ slides (Menzel-Gläser). Slides were either processed immediately or stored in -20°C.

17. *In vivo* xenograft studies (III)

All experiments involving mice were approved by the National Animal Experiment Board (license number ESAVI/6285/04.10.07/2014). For the xenograft studies we first created stably luciferase expressing U87MG and U373MG cells. These cells were then further transduced to produce either NTN1FH or NTN1(II)FH. The cells were implanted into the brain of BALB/C NU/NU athymic mice as described earlier (Lee *et al.*, 2012; Ozawa and James, 2010). Five mice per cell-line were used. Intracranial implantation of the cells was performed as previously published. Shortly, 150 000 cells in 5µl of PBS were intracranially injected. The injection site was positioned 2 mm right and 1 mm anterior to the bregma suture and 2,5mm in depth. The tumor growth was followed with bioluminescence imaging. 3mg of D-Luciferin (Regis Technologies) in PBS was administered intraperitoneally to each mouse and allowed to circulate for 15 min. The photons emitted by the tumors were imaged using Perkin-Elmer IVIS 100 imaging system. The mice were sacrificed 21 days after U87MG implantations and 52 days after U373MG implantation. Their brain was collected fixed with 4% paraformaldehyde in PBS for 30 min. The tissue was then washed with PBS and incubated with 30% sucrose 24 hours. After that the tissue was embedded into OCT compound (Sakura Bioteh) in a cryomold (Sakura Biotech) and frozen using liquid nitrogen. The frozen tissue blocks were then stored in -80°C.

18. 3D ex vivo GBM tissue culture (III)

Fresh GBM tissue biopsies were washed with PBS and embedded into 50µl of Matrigel. The Matrigel was allowed to polymerize for 30 min in +37°C. DMEM/F12 cell culture medium supplemented with 100 IU/ml penicillin, 50 mg/ml streptomycin, B27 supplement, EGF 20ng/ml, FGF 20ng/ml was added on top of Matrigel drops. Tissue pieces were allowed to grow and invade for 7 days. Next, the Matrigel drops were transferred into cryomolds filled with OCT compound (Sakura Biotech) and fresh frozen with liquid nitrogen. 7µm thick sections were cut onto Superfrost slides. The tissue sections were then subjected to tissue immunofluorescence analysis.

19. Establishing primary GBM cell lines (III)

Primary GBM cultures were established according to a previously published protocol (Hasselbach *et al.*, 2014b). Briefly, fresh GBM tissue biopsies were minced and cells detached by with trypsin and vigorous mixing. Trypsin was inhibited and cells collected by centrifugation. The number of viable cells was determined with Trypan blue (Bio Rad). The cells were diluted into density of 50 000 cells/ml into DMEM/F12 cell culture medium supplemented with 100 IU/ml penicillin, 50 mg/ml streptomycin, B27 supplement, EGF 20ng/ml, FGF 20ng/ml. The medium was changed twice a week and cells passaged every second week.

20. Co-culture of GSCs and U251MG cells in 3D Matrigel (III)

First, a GFP encoding pLenti CMV GFP Puro plasmid (Addgene) was lentivirally transduced into stably NTN1FH and NTN1(II)FH expressing U251MG cells. The stable GFP expression was ensured with flow cytometry based cell sorting. Next, the NTN1 and GFP expressing U251MG cells were mixed with primary GBM cells and allowed to form spheroids in non-adherent agarose coated U-bottom plates. Five spheroids were then mixed with 50µl Matrigel and plated on cell culture plates. The Matrigel was allowed to polymerize for 30 minutes and cell culture medium was added to the wells. The spheroids was imaged under Cell-IQ imaging system (Chip Man Tehcnologies) for 24 hours. The growth of the spheroids was analysed by measuring the area of the cells in different timepoints using the Cell IQ image analysis software (Chip Man Tehcnologies). The spheroids were fixed with 4% PFA 30min, permeabilised with 0,3% Tx100 PBS and subjected to immunofluorescence staining.

21. Statistical analysis (I, II, III)

Results are represent as the mean of at least three independent repeats. Error bars represent the standard deviation or standard error of mean. Statistical significance was analyzed with non-parametric Mann–Whitney U-test for independent samples.

22. Bioinformatics analysis (I, II)

Exon array data of 425 primary GBM samples and 10 normal brain tissues were obtained from The Cancer Genome Atlas (TCGA) (Cancer Genome Atlas Research Network, 2008). Preprocessing was performed at the gene expression level using the Multiple Exon Array Preprocessing (MEAP) algorithm (Chen *et al.*, 2011). Subsequent data analyses were performed using the Anduril framework (Ovaska *et al.*, 2010). Candidate pathways were investigated using the Moksiskaan database to determine comprehensive signaling pathway networks from canonical pathways (Laakso and Hautaniemi, 2010). To analyze the survival of patients, 393 samples that had survival end time in the TCGA GBM repository were used. Fold change (FC) values were calculated by taking the median and used in grouping for Kaplan-Meier survival analysis, where patients were divided into groups denoted by "-1" (underexpression, $FC < 0.5$), "1" (overexpression, $FC > 3$), and "0" (stable expression). Survival *P* values were calculated with the log rank test, and the threshold used in our analysis was *P* $< .05$.

RESULTS

1. NTN4 promotes GBM cell proliferation and motility in a concentration dependent manner (I)

Previously high expression of NTN4 had been shown in normal human astrocytes (Staquicini *et al.*, 2009). To investigate the effects of NTN4 in GBM cells we utilized three glioma cell-lines: U87MG, U251MG and U373MG. Interestingly, all studied glioma cell lines did express NTN4 but less than normal astrocytes. To characterize the effects of NTN4 on the GBM cells its expression was silenced using lentivirus mediated shRNA delivery. The silencing of NTN4 led to the decrease of cell proliferation in BrDU incorporation assay, decreased cell migration in wound closure assay and increased apoptotic labeling in TUNEL assay. Furthermore, exogenous addition of recombinant NTN4 increased cell proliferation and migration. However, the additive effect was concentration dependent: addition of 50ng/ml and 200ng/ml increased the proliferation and migration while addition of 5000ng/ml did not affect or even suppressed proliferation and migration. Similar findings were observed with U373MG whereas in U87MG cells NTN4 induced effects were limited to cell proliferation.

2. NTN4 stimulates GBM cell proliferation via ITGB4 dependent AKT-mTOR phosphorylation (I)

Tandem affinity purification and mass spectrometry screen demonstrated that NTN4 interact with ITGB4. This observation was validated using immunoprecipitation and immunofluorescence analyses. Interestingly, examination of the TCGA database revealed that the expression levels of NTN4 and ITGB4 are opposite in GBM tissue: NTN4 was downregulated whereas the expression levels of ITGB4 were upregulated. However, decrease in NTN4 and increase in ITGB4 were both associated with poor patient survival.

AKT, mTOR and insulin receptor substrates are mediators of ITGB4 signaling (Nikolopoulos *et al.*, 2004; Shaw, 2001). In our screen, insulin receptor substrates were also identified to interact with NTN4. Previously, it has been shown that NTN is connected to AKT signaling pathway in tumor cells (Nacht *et al.*, 2009). In addition, mTOR signaling has been previously linked to cell proliferation (Shaw and Cantley, 2006). Further investigation revealed that NTN4 silencing decreased phosphorylation of AKT, mTOR and ERK1/2 whereas exogenous addition of 0-50ng/ml recombinant NTN4 increased it. Silencing of ITGB4 abolished this effect suggesting that it is an essential mediator of NTN4 induced AKT-mTOR phosphorylation.

3. NTN1 promotes GBM cell invasiveness *in vitro* and *in vivo* (II, III)

We then went on to characterize the expression levels of NTN1 in three GBM cell lines: U87MG, U251MG and U373MG using quantitative western blotting and qRT-PCR. Interestingly, when compared to human normal astrocytes the expression of NTN1 was upregulated in invasive GBM cell lines U251 and U373MG whereas in solid tumor forming U87MG the expression was downregulated.

Intrigued by these findings we hypothesized that NTN1 is a regulator of GBM invasion. We first tested this on cell-lines in vitro. In a 2D Matrigel invasion assay NTN1 overexpression

increased U251MG cell invasion whereas lentivirus mediated shRNA knock-down reduced it. The reduction was observed in a manner corresponding to the efficiency of the silencing. Complete silencing led to the apoptotic death of the cells (unpublished data). The role of NTN1 in the GBM invasiveness was further deciphered in an *in vivo* xenograft model. First, NTN1 overexpressing or wildtype U87MG cells were orthotopically xenografted into nude mice. The cells were expressing firefly luciferase to allow tumor growth monitoring by bioluminescence imaging. NTN1 expression resulted in more widely spread tumor and stronger bioluminescent signal.

After sacrificing the mice the growth of the tumors was assessed on tissue sections of the brain. Consistently with the bioluminescence imaging tumors formed by NTN1 overexpressing cells were larger and more invasive. The invasive fronts of the NTN1 expressing tumor were less well-defined and single tumor cells had started to spread to the brain parenchyma along the blood vasculature. These findings confirm the role of NTN1 as invasion promoting factor in GBM.

4. NTN1 is a multifunctional protein which associates with several proteins and signaling pathways (II)

The proteins interacting with NTN1 were screened using tandem affinity chromatography and identified with mass spectrometry. For the purification, all NTN1 domains (N-terminal domain = NTN(I)FH; central domain = NTN1(II)FH; C-terminal-domain = NTN1(III)FH) were expressed separately in U251MG cells and tagged with FLAG and HA tags for the pull down. The different domains served as internal controls for each other and also provided information on the roles of the different domains.

Our screen was validated by the identification of two known NTN1 receptors ITGA3B1 and ITGB4 (Yebra *et al.*, 2003). The pathways that NTN1 was associated with were identified using automated pathway analysis that is based on Kyoto Encyclopedia of Genes and Genomes. It revealed that NTN1 associated with various cell signaling pathways including ECM-receptor interaction, insulin signaling and TGF-beta signaling pathway. However, out of the identified pathways the most prominent candidate pathway was Notch signaling pathway. NTN1(I)FH interacted with Notch2 and Notch3 receptors, NTN1(II)FH with Notch1 and Notch2 receptors and NTN1(III)FH with Notch2 receptors. NTN1(II)FH also interacted with known the Notch ligand Jagged1.

Protease mediated cleavage of the Notch receptor and the endocytosis of the intracellular domain of the receptor are important parts in the activation of the Notch signaling (reviewed in Parks *et al.*, 2000; Pratt *et al.*, 2011). Interestingly, different NTN1 domains interacted with several proteins which may be related to the Notch signaling activation. NTN1(I)FH interacted with several endocytosis related proteins, NTN1(II)FH interacted with E3 ligases and NTN1(II)FH with multiple proteases. These results suggest that the domains may independently modulate the Notch signaling.

5. NTN1 can activate Notch signaling and induce subsequent cell invasion by binding to the Notch-Jagged complex (II)

The binding of NTN1 and Notch signaling components was validated by immunoprecipitation and western blotting. Indeed all the domains could pull-down Notch2 and Jagged1 from U251MG cell lysate. However, the pull-down was most efficient with full-length NTN1 and NTN1(II)FH. The interaction was further analyzed using immunofluorescence microscopy. U251MG cells were treated with NTN1FH containing medium either at 37°C or at +4°C. When the treatment was performed at +4°C NTN1FH colocalized strongly with Jagged1 on cell-cell contacts. At +37°C, NTN1FH and Jagged1 colocalized in vesicle-like structures suggesting that they are endocytosed together. On the other hand, NTN1FH colocalized with Notch2 on cell surface in both temperatures.

The ability of NTN1 to modulate Notch signaling was investigated using a Notch reporter assay. A construct where Notch response element controlled luciferase coding gene was transiently transfected into either NTN1 overexpressing or NTN1 silenced U251MG cells. NTN1 upregulation induced over 2-fold increase in Notch activation in comparison to wildtype U251MG cells. Consistently, NTN1 silencing decreased Notch signaling significantly.

Furthermore, we also examined how Notch signaling inhibition affects the invasiveness of GBM cells. We utilized DAPT, a compound that prevents the γ-secretase mediated cleavage of Notch receptor which is essential for the activation of the signaling. U251MG cells were used in Matrigel invasion assay with or without DAPT. Upon DAPT treatment the invasive capability of the cells was markedly reduced.

Taken together these results suggest that NTN1 can bind to the signaling complex of Notch2 and Jagged1 and activate the Notch signaling. NTN1 is endocytosed together with Jagged1 plausibly after the cleavage of the Notch receptor. Furthermore, inhibition of Notch signaling with DAPT inhibited cell invasion similarly to NTN1 suggesting that it is mediating NTN1 effects.

6. The expression of NTN1 in human glioma biopsies is associated with poor patient prognosis (II, III)

The expression of NTN1 in human gliomas was analyzed both on gene and protein expression levels. TCGA exon array datasets revealed that the expression of NTN1 was markedly upregulated in GBM samples compared to normal brain. Next, the NTN1 expression was assessed in a tissue microarray of 136 tissue samples which included astrocytoma, anaplastic astrocytoma, oligodendroglioma, oligoastrocytoma and glioblastoma tumors (Sihto *et al.*, 2007; Tynninen *et al.*, 2004). NTN1 was expressed in all glioma subtypes. Within all the tissue samples 40.4% of the tumors were NTN1 positive. NTN1 was strongly associated with astrocytic tumors. Within astrocytomas 77.8%, within anaplastic astrocytomas 62.5% and within GBMs 77.1% of the tumors were NTN1 positive whereas within oligodendrogliomas and oligoastrocytomas only 26.7% and 36.8%, respectively, were NTN1 positive. No association with gender or age at the time of diagnosis was observed.

Furthermore, we investigated how NTN1 expression in gliomas was related to the survival of the patients and performed Kaplan-Meier survival analysis. Both glioma-specific overall and recurrence-free survival times were analyzed. Interestingly, NTN1 positivity was associated with poor glioma specific survival (hazard ratio [HR] = 1.73, 95% confidence interval [95% CI]

= 1.11 to 2.71; p=0.015) and shorter recurrence-free survival time (HR = 1.62, 95% CI = 1.04 to 2.53; p<0.001) (Fig. 1B). However, when astrocytomas and oligodendroglial tumors were analyzed separately NTN1 expression was not significantly associated with patient survival.

7. NTN1 locates to GSC-rich tumor areas and to the stalk area of GSC led invasive structures (III)

Next, we analyzed the localization of NTN1 in glioma tissue. In all glioma subtypes NTN1 was enriched to areas surrounding the necrotic tumor core and especially to pseudopalisade structures that contain actively migrating cells (Brat *et al.*, 2004a) in GBM samples. Also the abnormal glomerular vessels of GBM tumors were positive for NTN1.

The hypoxic and perivascular GBM tumor areas where NTN1 was located are known to be rich in GSCs (Bar *et al.*, 2010; Bar *et al.*, 2010; Calabrese *et al.*, 2007; Heddleston *et al.*, 2009). While we were deciphering the expression of NTN1 in the glioma TMAs we also observed that NTN1 and nestin expressions correlated. Previously, nestin has been connected to the stemness of the GBM cells and to the poor prognosis of glioma patients (Strojnik *et al.*, 2007; Zhang *et al.*, 2008). In addition, Notch signaling has been observed to promote the stemness of the GBM cells (Chen *et al.*, 2010; Fan *et al.*, 2010; Hu *et al.*, 2011). Therefore, we hypothesized that NTN1 affects the GSCs via Notch signaling. To explore this further we examined whether NTN1 co-localizes with known GSC markers nestin and CD133 in fresh frozen GBM tissue. Interestingly, we did not observe co-localization of NTN1 in same cells with either of these markers. Instead, NTN1 localized to neighboring cells of nestin expressing cells. Similar localization was observed with CD133. In the GBM tissues there were areas with CD133 positive cells surrounded by NTN1 positive cells. Next, we investigated the co-localization of NTN1 and Notch2 receptor or Jagged1 ligand on the GBM tissue. We observed that NTN1 and Jagged1 co-localized on the same cells in a manner similar to what we observed in *in vitro* cultured GBM cells. In contrast, NTN1 and Notch2 were not co-localized in same tumor cells. Instead, we observed that Notch2 and NTN1 were expressed in neighboring cells and were co-localized within their cell-cell contacts.

We also analyzed the NTN1 and nestin localization in the U87MG xenografts. Expression of CD133 could not be studied because the U87GM cells do not express it. We stained the NTN1FH tumor sections for nestin and HA to localize NTN1 positive cells in the tumors. Unexpectedly, we detected that the invasive edge was strongly nestin positive in NTN1 overexpressing tumors but not in control tumors. Furthermore, we observed that the invasive structures consistently showed an assembly where the nestin positive cells were on the leading edge whereas NTN1 positive cells were following them. We also observed similar pattern with Notch2 staining. Again, the NTN1 positive cells were in the stalk area of the invasive sprouts whereas Notch2 positive cells were in the invasive front.

Because these results link NTN1 strongly to GBM invasiveness and stemness we were interested in examining its localization in the invasive cells of human GBM too. However, the surgical human GBM biopsies represent primarily the tumor core because the single invasive cells are impossible to remove upon surgery. To mimic the invasive front of human GBM we established ex vivo human GBM cultures. We implanted freshly collected GBM biopsies in 3D Matrigel and allowed the cells to grow and migrate for 7 days. The Matrigel plugs were then fresh frozen and sectioned. Immunofluorescence staining of the sections revealed similar pattern that was observed with the xenografts. The front of the invasive structures

was positive for Notch2 and for nestin suggesting that these cells are more stem-like than the cells in tumor core. Again, NTN1 positive cells remained at the stalk area of the invasive sprouts. These findings are consistent with the observations of the U87MG xenograft model where the leading cells of the invasive structures were nestin and Notch positive whereas NTN1 positive cells remained in the stalk area of the structures.

8. NTN1 increases the number of GSCs in the cell population and stimulates their motility (III)

To analyze how NTN1 affects the GSCs, we cultured both wild type and NTN1 overexpressing U251MG GBM cells under conditions that favor neural stem cell proliferation (Wakimoto *et al.*, 2012). Interestingly, NTN1 overexpression led to 16-fold higher neurosphere formation compared to wild-type cells. These neurospheres also expressed stemness markers Sox2, nestin and integrin alpha6 more than normal U251MG cells. This suggests that the initial NTN1 overexpressing cell population contained a higher number of GSCs and that NTN1 plays a role in their maintenance.

Next, we investigated how NTN1 affects the motility of the GSCs and used 2D and 3D invasion assays. The U251MG GSCs were either plated as single cells on top of 2D Matrigel or the neurospheres as such were implanted into 3D Matrigel. 50ng/ml of recombinant NTN1 (rNTN1) was utilized as attractant in the assays. In both assays the addition of rNTN1 enhanced the motility of the cells: they either invaded through the Boyden chamber more effectively or the neurospheres spread much faster upon rNTN1 addition. Furthermore, the control spheroids remained more compact compared to rNTN1 treated, which spread more diffusively. These results suggest that rNTN1 regulates the motility of GSCs.

9. NTN1(II)FH diminishes the activation Notch signaling by retaining Notch-Jagged1 signaling complex on cell surface (II)

While we were validating the mass spectrometry results we observed that both the full-length NTN1 and its central domain (NTN1(II)FH) bound to Notch2 and Jagged1 most efficiently. Therefore, we included NTN1(II)FH into our further analysis too. Immunofluorescence analysis revealed that NTN1(II)FH also co-localized with both Notch2 and Jagged1 on cell surface but it was not internalized with either of them. Surprisingly, its effect to the Notch signaling pathway was opposite than the full-length NTN1: NTN1(II)FH decreased the activation whereas NTN1 activated it. Furthermore, it modulated the morphology of the cells towards a smaller and more rounded phenotype. Similar effect has been observed upon Notch signaling inhibition with DAPT (Ingram *et al.*, 2008). Because the U251MG cells express NTN1 endogenously, our results suggest that NTN1(II)FH can overcome the effects of the full-length protein.

We then analyzed whether the defect in Notch signaling was due to the altered cell surface localization of either Notch2 or Jagged1. We biotinylated all cell surface proteins and visualized the cell surface fraction of Notch and Jagged with immunoprecipitation and immunoblotting. Both Notch2 and Jagged1 were similarly expressed on the surface of wildtype U251MG cells or stably NTN1 or NTN1(II)FH expressing U251MG cells. Surprisingly,

we observed that upon NTN1(II)FH expression Notch2 could more effectively immunoprecipitate Jagged1 from the cell surface. Furthermore, the co-localization of Notch2 intracellular domain and clathrin on cell membrane was decreased. Because Notch internalization which is required for the signaling activation occurs via clathrin mediated endocytosis (Windler and Bilder, 2010) our findings suggest that NTN1(II)FH decreases the signaling by retaining the Notch signaling complex on cell surface.

10. Inhibition of NTN1 signaling with NTN1(II)FH reduces GBM invasiveness via decreasing the motility of GSCs (II, III)

Since NTN1(II)FH had the opposite effect on Notch signaling compared to full-length NTN1 we were interested in testing how it affects cell invasiveness. We first studied this *in vitro* in 2D Matrigel invasion assay. As expected, the expression of NTN1(II)FH decreased the invasiveness of U251MG cells. Next, the effects of the NTN1(II)FH were evaluated *in vivo*. We performed intracranial xenografts in nude mice and used either wildtype, NTN1FH expressing or NTN1(II)FH expressing U373MG cells. The U373MG cells were employed because they endogenously express NTN1 and thus were a good model to study NTN1 inhibition. Again, the tumor growth was monitored using bioluminescence imaging. After 6 weeks we started to observe differences in the tumor growth between the groups. Both the control tumors and NTN1FH tumors grew all around the mouse head area and also spread along the spine of the mice whereas the growth of the NTN1(II)FH tumors was restricted to the head area. When we measured the emitted photons only from the head area of the mice we could not observe any significant difference between the groups. However, combining the signal from the head and the spine of the mice revealed a significant decrease on the photons within NTN1(II)FH group. These results suggest that NTN1(II)FH can antagonize the effects of endogenously expressed NTN1.

Next, we wanted to analyze does NTN1(II)FH affect the motility of the GSCs. Freshly isolated GBM cell-lines that are kept under the stem-like cell proliferation favoring culture conditions are a better model for investigating glioma stem-like cells than long cultured cell (Ahmad *et al.*, 2014; Wakimoto *et al.*, 2012). Therefore, we established primary GSC cultures from surgical GBM biopsies, named GBM9 and GBM10, which were constantly cultured under stem-like cell proliferation favoring conditions. Because GBM tumors are heterogeneous and contain both GSCs and differentiated tumor cells we mixed the GBM9 or GBM10 cells with NTN1 expressing and GFP labelled U251MG cells. The U251MG cells cultured under adherent conditions mimicked the differentiated GBM cell population in the human tumors. These mixture spheroids were then embedded into 3D Matrigel matrix.

We observed that once the GBM9 cells were mixed with control U251MG cells or NTN1FH expressing U251MG cells (GBM9/U251MG and GBM9/U251MG-NTN1FH spheroids respectively) the spheroids invaded diffusively and presented two types of sprouts: sprouts where the GFP positive U251MG cells were leading and where GFP negative GBM9 cells where leading. On the contrary, the GBM9 and U251-NTN1(II)FH spheroids (GBM9/NTN1(II)FH) invaded in more compact manner and presented only GFP positive sprouts. We quantified the sprouts and indeed in GBM9/U251 and in GBM9/U251-NTN1FH spheroids both sprout types existed in equal proportions. In GBM9/U251MG-NTN1(II)FH spheroids more than 80% of the sprouts were led by U251GM cells. Consistent results were obtained with GBM10 and U251MG cell mixtures.

Immunofluorescence staining with nestin and HA to recognize NTN1 confirmed that GBM9/U251-NTN1FH spheroids presented similar GSC led sprout assembly than in the xenograft and *ex vivo* models. In addition, the cells leading the invasion were positive for cleaved-Notch suggesting activated Notch signaling. In contrast, the sprouts led by the nestin positive and Notch-activated cells were lacking in the GBM9/U251-NTN1(II)FH spheroids. Taken together these results suggest that NTN1 regulates the GBM invasiveness by promoting the motility of the GSCs and the inhibition of the NTN1 signaling with NTN1(II)FH peptide reduces the invasiveness of especially GBM stem-like cells.

DISCUSSION

1. NTN4 regulates the proliferation of glioma cells via orchestrated actions of ITGB4 and UNC5B (I)

NTN4 is a multifunctional protein that has been linked to many cancers. However, its role is controversial since some reports have identified it as cancer promoter and others as a tumor suppressor. Here we have investigated its effects and signaling in glioblastoma. We observed that it modulates glioblastoma cell motility, survival and proliferation in a concentration dependent manner: low concentration promoted these effects whereas high concentrations inhibited them. Similarly NTN4 regulates adhesion, migration, proliferation and apoptosis of endothelial cells in a concentration dependent manner (Larrieu-Lahargue *et al.*, 2010; Lejmi *et al.*, 2008; Wilson *et al.*, 2006). Furthermore, we observed that its expression is reduced during glioma progression and that low NTN4 expression is correlated with poor patient prognosis. Taken together, these findings provide evidence that NTN4 plays a dual function in glioma progression and that its effects depend on a balance of its expression. These results could also explain the previous contradicting results in other cancers.

To better understand how NTN4 regulates glioma cells we screened for pathways mediating its effects. We discovered that ITGB4 binds to NTN4 and mediates the positive effects of NTN4 on glioblastoma cell proliferation. Previously ITGA2B1 and ITGA3B1 have been recognized as NTN4 receptors (Maedler *et al.*, 2011). Furthermore, ITGB4 is expressed strongly in astrocytes, and it is upregulated during glioma progression (Previtali *et al.*, 1996). Consistently, we observed that ITGB4 upregulation is associated with poor patient survival in GBM.

Interestingly, suppression of NTN4 expression in ITGB4-silenced cells slightly increased cell proliferation, and NTN4 overexpression led to decreased proliferation in ITGB4- silenced glioblastoma cells. These results suggest that there are other mechanisms mediating the negative effects of NTN4. Previously, UNC5B has been observed to mediate NTN4 induced inhibitory functions of endothelial cells (Lejmi *et al.*, 2008). Therefore, we analyzed whether UNC5B could be the receptor mediating the negative effects also in GBM. Unlike NTN4 and ITGB4 the expression of UNCB5 was not altered in GBM when compared to normal brain. Silencing of UNC5B led to decreased proliferation of GBM cells. Addition of high concentration of NTN4 did not further suppress the proliferation suggesting that UNC5B is mediating this effect.

Our findings elucidate a complicated mechanism by which NTN4 regulates the proliferation of GBM cells. When there is excess supply of NTN4 available it binds to UNC5B which then suppresses cell proliferation. NTN4 also binds to ITGB4 that is expressed in low levels and induces proliferation. This balance maintains the glial cell proliferation on a sufficient level for normal growth. However, during tumor progression the expression level of ITGB4 gets upregulated and NTN4 gets downregulated while the level of UNC5B remains unchanged. The relatively low number of NTN4 proteins bind efficiently to ITGB4 due to its abundant expression on cell surface. Since majority of the NTN4 binds to ITGB4 the UNC5B mediated proliferation inhibition is lost and ITGB4 signaling upregulate GBM cell proliferation.

2. NTN1 drives the GBM invasion and stemness via Notch signaling (II, III)

Like NTN4 also NTN1 regulates several cancers. It acts as invasion promoting factor in pancreatic, colorectal and hepatocytic cancer and in medulloblastoma (Akino *et al.*, 2014; Dumartin *et al.*, 2010; Rodrigues *et al.*, 2007; Yan *et al.*, 2014). Interestingly, NTN1 has been suggested to function also as a potential biomarker in both glioblastoma and medulloblastoma (Akino *et al.*, 2014; Ramesh *et al.*, 2011). In this thesis, we link NTN1 to glioblastoma invasion and stemness. The expression of NTN1 is high in glioblastoma derived cell-lines that show infiltrative growth *in vivo* but not in less malignant cells. In a TMA screen of various glioma biopsies, NTN1 associated strongly with astrocytomas which are the most invasive form of gliomas (Palfi *et al.*, 2004). Furthermore, NTN1 expression was associated with poor patient prognosis. NTN1 upregulation promoted the invasiveness of glioblastoma cells *in vitro* and *in vivo.* Consistently with our findings, another study also found that NTN1 promotes the GBM invasiveness (Shimizu *et al.*, 2013).

To elucidate the signaling mechanisms mediating NTN1 effects we systematically screened its binding partners using tandem affinity purification and mass spectrometry protein identification. Out of the identified receptors, Notch pathway was the most prominent. NTN1 physically interacted with Notch pathway components: NTN1 pulled down both Notch2 and Jagged1 and colocalized with both Jagged1 and Notch2 on the cell surface. However, NTN1 colocalized with Jagged1 also in cytoplasm but not with Notch2. Moreover, NTN1 enhanced Notch activity. Both NTN1 and Notch signaling induced GBM cell invasion. Taken together, these findings suggest that NTN1 induced cell invasion is mediated by Notch signaling. NTN1 can bind to the complex of Notch and Jagged and activate the signaling.

Previously, Notch signaling has been associated with increased GBM invasion (Chigurupati *et al.*, 2010; Sivasankaran *et al.*, 2009; Stockhausen *et al.*, 2010; Zhang *et al.*, 2012) and maintenance of the GSCs within GBM tumors (Chen *et al.*, 2010; Fan *et al.*, 2010; Hu *et al.*, 2011) and to the upregulation of nestin expression (Shih and Holland, 2006). In addition, NTN1 has been linked to the up-regulation of stem cell motility and self-renewal during developmental processes (Lee *et al.*, 2014; Ozmadenci *et al.*, 2015). Therefore, we speculated that the NTN1 induced upregulation of Notch signaling could promote the stemness of the glioma cells. Indeed, we observed that when NTN1 was overexpressed in GBM cells *in vitro* their percentage of GSCs was increased. This suggests that NTN1 promotes the maintenance of the GSCs within the population. Similarly to cultured cells, NTN1 colocalized with Jagged1 but not Notch2 in human GBM tissue. However, Notch2 and NTN1 did colocalize on the cell-cell contacts of neighboring cells. Similar coexpression in neighboring cells was observed with NTN1 and CD133 and nestin. Furthermore, we observed that in the NTN1 expressing U87MG xenografts tumors the invasive colonies or sprouts were growing in a distinct assembly. The cells in the leading edge were positive for nestin and Notch2 whereas the cells in the stalk area of the sprouts were NTN1 positive. Similar structures were visible in both *in vivo* xenograft models and in *ex vivo* human GBM tissue cultures. The structure of these sprouts was similar to those observed in NTN1 guided lung and mammary gland morphogenesis (Dalvin *et al.*, 2003; Liu *et al.*, 2004).

Taken together, these finding describe a new mechanism of GBM invasiveness. NTN1 in the stalk area of the invasive structures can activate the Notch signaling in the adjacent cells that form the leading edge of the sprouts (Fig. 5). This allows the maintenance of stem like

characteristics of the leading cells which is a great advantage because the stem-like cells are very plastic and motile.
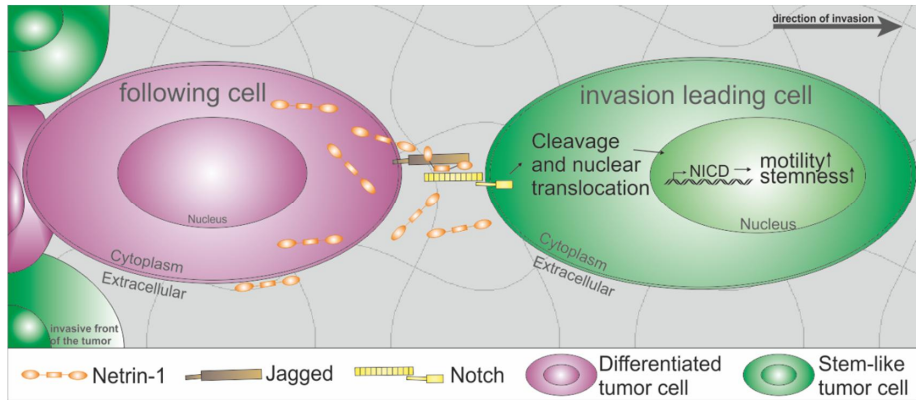

Figure 5. Model of the NTN1 induced invasiveness of the GBM cells. This schematic illustration summarizes the main findings of the articles II and III. NTN1 in the stalk area of invasive sprouts activates Notch signaling in the leading edge. Modified from (Ylivinkka *et al.*, 2017).

GBM tumors are heterogeneous and contain different regions. Besides the invasive front, the GSCs are located to the hypoxic and perivascular tumor areas (Bar *et al.*, 2010; Bar *et al.*, 2010; Calabrese *et al.*, 2007; Heddleston *et al.*, 2009). NTN1 is enriched to these same areas and was located next to GSCs within the tumor core. Furthermore, NTN1 secretion is upregulated under hypoxia (unpublished results). Since NTN1 and Notch can promote the maintenance of GSCs this may give them and advantage to survive within the tumor core. Furthermore, NTN1 induced apoptosis resistance is shown to protect the embryonic stem cells (Ozmadenci *et al.*, 2015). Therefore, NTN1 may have similar GSC protective mechanisms in GBM too.

3. NTN1(II)FH overcomes the effects of full-length NTN1 and has therapeutic potential (II, III)

Currently, no curative treatment exists for GBM. The only approved drug, TMZ, provides only modest prolongation of the lifetime of the patients (Venur *et al.*, 2015). Therefore, the discovery of new treatment options and enhanced targeting of the existing treatments are vital. Infiltrative invasiveness and the stemness of the tumor cells are hallmarks of GBM and also the main reasons for the treatment failure (Ortensi *et al.*, 2013). Our results indicate that NTN1 is a novel regulator of GBM invasiveness and stemness. By inhibiting NTN1 signaling both of these phenotypes could be targeted suggesting NTN1 as potential treatment target in GBM.

Interestingly, NTN1(II)FH, a peptide consisting of the central domain of NTN1, specifically counteracts NTN1–Notch signaling as indicated by several results: NTN1(II)FH was able to pull down both Notch2 and Jagged1. It colocalized with Notch2 and Jagged1 at the cell surface but not in the cytoplasm. Exogenous NTN1(II)FH also altered the morphology of U251MG cells similarly to the Notch signaling inhibitor DAPT. The Notch2–Jagged1 signaling complex was retained at the cell surface and less colocalization of Notch2 and clathrin was

observed on the cell surface in the presence of NTN1(II)FH suggesting inhibition of the signaling. Furthermore, NTN(II)FH expression inhibited the invasiveness of the GBM cells *in vitro* and *in vivo.* This reduction in invasiveness was due to the decreased motility of GSCs. These results suggest that NTN1 signaling inhibition could offer a powerful way to target GBM and that NTN1(II)FH provides one possible way for targeting that.

The Notch inhibitors have been actively investigated as a possible therapeutic for GBM. Currently two phase I/0 clinical studies have been conducted (Krop *et al.*, 2012; Xu *et al.*, 2016). The first one included patients with different late stage solid tumors (Krop *et al.*, 2012) and the other was designed to evaluate the biological effects of the inhibitor (Xu *et al.*, 2016). Therefore, the patient benefit is difficult to estimate based on these studies. However, the first study did observe that only the glioma patients responded to the treatment even though results were not dramatic. This suggests that Notch inhibition could be effective in GBM treatment.

Both of these clinical studies utilize GSIs which prevent the Notch activation due to the lack of γ-secretase mediated receptor cleavage. However, it has been suggested that alternative, γ-secretase independent ways to activate the signaling exist (Ayaz and Osborne, 2014; Baron, 2012). Therefore, it is possible that the GSI targeted treatments do not totally prevent Notch signaling activation. In this thesis we describe a novel NTN1 induced mechanism for Notch activation. Furthermore, according to the mass spectrometry results NTN1 can bind to various Notch receptors. Here, we have employed Notch2 as a model receptor due to its abundant expression in GBM cells. However, the NTN1 induced Notch activation may not be limited to Notch2. Therefore, the described NTN1-Notch signaling inhibition with NTN1(II)FH peptide may be of great clinical importance.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis we describe two mechanism how NTNs modulate GBM pathogenity. First, we described a mechanism of NTN4-ITGB4 that drives the proliferation of glioma cells during the tumor progression (I). Second, we described how NTN1 promotes glioma cell invasiveness and stemness via Notch signaling (II, III). Based on these findings, NTNs could be interesting therapeutic targets. Inhibition of their signaling could inhibit both cell proliferation and cell invasion, two major mechanisms leading to lethality of GBM. In addition, we link NTN1 to tumor cell stemness which not only affects cell invasion but also resistance to therapies. Furthermore, we designed a peptide that can inhibit NTN1 signaling (II, III). This peptide has therapeutic potential: it can target both cell invasion and stemness that are key hallmarks of gliomas.

For future studies, our results raise several questions regarding the role of NTN1 in glioma biology. During brain injuries, NTN1 is upregulated within the SVZ (Cayre *et al.*, 2013). There it increases the motility of neural progenitors and promotes angiogenesis to create new paths for neural precursor migration (Cayre *et al.*, 2013). Similarly, the glioma cells use the vasculature as tracks for invasion. We observed in our xenograft models that the invasive cells migrating from NTN1 overexpressing xenografts were co-opting the surrounding vasculature. Therefore it is interesting to speculate that NTN1 could promote tumor vasculature to create highways for the GSCs.

Furthermore, the cell of origin of GBM is still unclear. It has been postulated that it could be a transformed neural stem or precursor cell raising from the SVZ (Alcantara Llaguno *et al.*, 2009). Since NTN1 is expressed within the SVZ and it is capable of maintaining the GSCs it raises the question whether it participates to the initiation of the GBM. Moreover, it is unclear whether the GBM tumors consist of two different cell populations: the GSCs and the differentiated tumor cells or whether the stemness is an intrinsic property of all GBM cells. Our results indicate that NTN1 can modulate the plasticity of the GBM cells. It altered both U251MG and U87MG cell populations towards more stem-like phenotype suggesting that it may play a role in the regulation of GBM heterogeneity. However, it still remains unclear whether it increases the number of GSCs or whether it can reprogram the differentiated GBM cells towards more stem-like phenotype.

Besides the mechanisms we have studied in detail in this thesis, our results implicate that NTNs are involved in many other signaling pathways. We screened for interacting partners of both NTN1 and NTN4 and discovered several signaling pathways and cellular functions connected to them. For example, the identified interacting partners of NTN1 included integrin signaling, proteolysis and endocytosis related proteins. Even though some interacting proteins were shared between the NTN1 fragments, each of them also had unique binding partners suggesting that NTN1 can modulate various pathways via the different domains. Besides GBM that was used as a model in these studies, NTNs have been linked to other cancers and other pathological conditions such as neurological diseases. Many of the signaling pathways discovered here may be of interest within these conditions too. Therefore, our results open many new research avenues and have implications beyond GBM.

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