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DISSERTATION

**Interaction of glioma cells and intrinsic brain cells-  
soluble factor mediated**

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Von

**Min-Chi Ku**

aus Keelung, Taiwan

Gutachter/in:

1. Prof. Dr. Helmut Kettenmann
2. Prof. Dr. Bozena Kaminska-Kaczmarek
3. Prof. Dr. med. Frank Heppner

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## Abbreviations

Blood-brain barrier (BBB)

Bovine serum albumin (BSA)

Bromodeoxyuridine (BrdU)

Carbondioxide (CO<sub>2</sub>)

Central nervous system (CNS)

Copy DNA (cDNA)

Dimethyl sulfoxide (DMSO)

Desoxyribonucleic acid (DNA)

Dulbecco's Modified Eagle Medium (DMEM)

*exempli gratia* (latin: for example; e.g.)

Enhanced green fluorescent protein (EGFP)

Enzyme-Linked Immuno Sorbent Assay (ELISA)

Fetal calf serum (FCS)

Glia cell derived neurotrophic factor (GDNF)

Glial fibrillary acidic protein (GFAP)

High grade (HG)

Horseradish peroxidase (HRP)

Hollow fiber (HF)

Immunoglobulin (Ig)

Ionized calcium-binding adaptor molecule (Iba)

Magnetic resonance imaging (MRI)

Messenger RNA (mRNA)

Neural progenitor/precursor cell (NPC)

Neuron-glia 2 (NG2)

Nitric oxide (NO)

Normal goat serum (NGS)

Phosphate buffer saline (PBS)

Phosphate buffer saline-Triton X-100 (PBS-T)

Polymerase chain reaction (PCR)

Reactive oxygen species (ROS)

Regulated upon Activation - Normal T cell Expressed and Secreted (RANTES)

Reverse transcriptase PCR (RT-PCR)

Revolutions per minute (rpm)

Ribonucleic acid (RNA)

Room temperature (RT)

Small interfering RNA (siRNA)

Small hairpin RNA (shRNA)

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Stromal cell-derived factor 1 (SDF-1)

Subventricular zone (SVZ)

Wild type (wt)

World health organization (WHO)

# 1. Introduction

## 1.1. Intrinsic cells of the CNS

The central nervous system (CNS) consists not only of neurons but also about 50% of glial cells and other types of supporting cells in mammalian brain (Fig 1.1). The term “glial cell” denotes a broad category of cells that are made up by many other CNS cell subtypes. The major types of glial cells in the CNS are astrocytes, microglia and oligodendrocytes. Glial cells are believed to support the entire structure of the microenvironment together with endothelial cell lining; however, recent accumulating evidence showed that, glial cells have many other important functions for maintaining the homogeneous balance, such as regulation of neurotransmitters, ion homeostasis, detoxification, organizing information network, release of neuropeptides and neurotrophins and regulate neurogenesis (Jessen 2004). The CNS has long been thought as exempt from the effects of the immune system due to the physical barriers for protection and lack of drainage. But it is now clear that glial cells in the CNS respond to inflammation and injury in unique ways (Bechmann and Nitsch 2001; Martino and Pluchino 2007). In particular, microglia are the immunocompetent cells in the CNS executing similar functions like macrophages in the periphery. As happens in other organs, cells in the CNS also undergo natural cell death. Microglia can keep the CNS microenvironment clean by sensing and phagocytosing dead cell debris. In addition, when immune responses are generated within the CNS or from outside the CNS, microglia receive and pass on that response to other cells (Carson 2002; Rock, Gekker et al. 2004; Ghorpade, Gendelman et al. 2008).

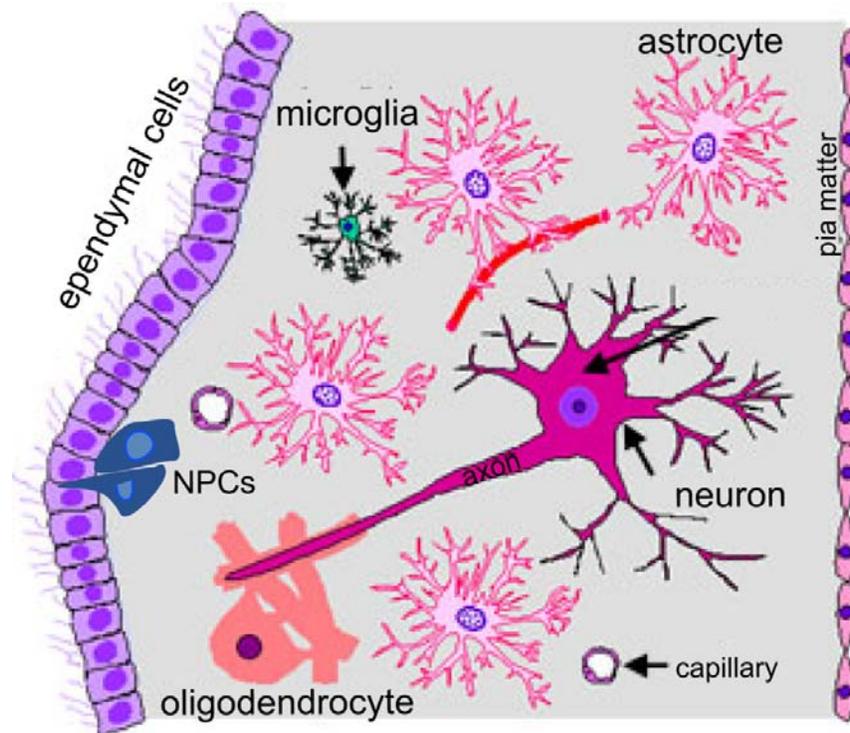
Another type of specialized glial cells are the ependymal cells that line at are at the interface between the brain parenchyma and the ventricular cavities. In addition to providing a barrier between the brain and the cerebral spinal fluid (CSF), ependymal cells are thought to function in secretion, absorption and transport of numerous molecules to isolate the potentially harmful substances in the CSF (Kuchler, Graff et al. 1994; Bruni 1998) and trigger neuronal differentiation/axonal guidance during development (Del Bigio 1995).

Astrocytes are found throughout the parenchyma and make up the largest glial population. Traditionally, they have been divided into protoplasmic astrocytes found mainly in the grey matter, and fibrous astrocytes present in both grey and white matter. Astrocytic end-foot processes with tight junctions contact the basal lamina of vessels and of the pia, contributing to the blood-brain and CSF-brain barriers. Functionally, unlike the rest of the body, where fibroblasts play the major role in scar formation, in the CNS, astrocytes play the major role in forming scar after traumatic injury. Astrocytes become reactive in response to CNS injury or disease accompany with blood brain barrier (BBB) breakdown, increased vascular permeability (Barres 2008). Major Concepts about astrocyte scar formation have long been

thought to be a pathological hallmark of CNS structural lesions due to induction of neurotoxicity and inflammation. By interpretation of scar formation it was the main impediment to functional recovery after CNS injury or disease. An recent review article pointed out that a large quantity of experimental studies suggested that reactive astrocyte also exerts essential beneficial functions such as helping to limit tissue degeneration and preserve function after CNS injury and does not even harm the CNS (Sofroniew and Vinters 2009). Thus, now it is believed that reactive astrogliosis exert both pro- and anti-inflammatory regulatory functions.

Oligodendrocytes are the third major population of glia and are ubiquitous throughout the adult CNS. In the white matter, oligodendrocytes produce myelin membranes that ensheath multiple axons and enable rapid impulse conduction. In the grey matter, oligodendrocytes may provide trophic signals to nearby neurons and synthesize growth factors. Oligodendrocytes express a wide variety of membrane ion channels and transporters such as glutamate transporter. Activation of glutamate receptor leads to the release of brain-derived neurotrophic factor which could promote myelin formation (Kolodziejczyk, Saab et al. 2010).

The adult mammalian CNS harbors neural progenitors, precursors, and stem cells that are capable of generating new neurons, astrocytes, and oligodendrocytes (Emsley, Mitchell et al. 2005). They have been found within certain specialized tissue compartments defined as 'germinal niches'. Subventricular zone (SVZ) and subgranular zone (SGZ) are now seen to be the major niches for neuronal precursor cells (NPC). Depends on different types of CNS injury, endogenous NPC might be beneficial to CNS repair owing to their ability to support neurogenesis and gliogenesis. NPC studies light up the future possibilities for development of novel neural repair strategies (Martino and Pluchino 2006).

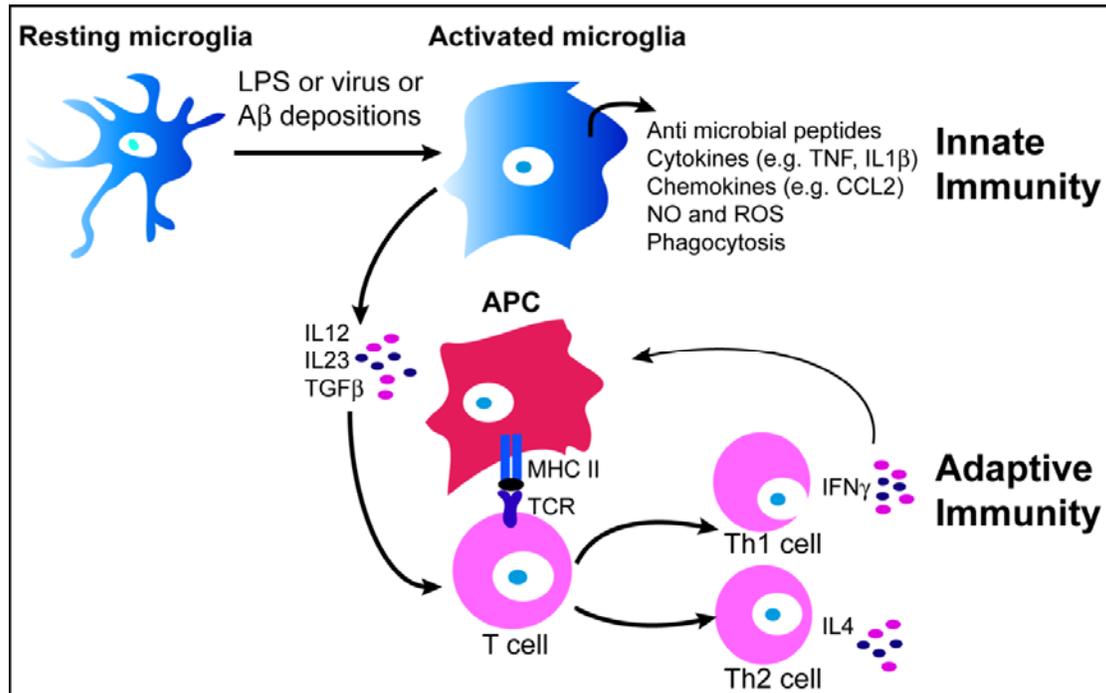


**Fig 1.1. The schematic view of major types of intrinsic CNS cells.** Mammalian CNS comprise neurons, the three major types of glial cells (astrocytes, microglia, and oligodendrocytes) and other supporting cell. Image modified from ([http://www.histology.leeds.ac.uk/tissue\\_types/nerves/Nerve\\_support\\_cell.php](http://www.histology.leeds.ac.uk/tissue_types/nerves/Nerve_support_cell.php))

### 1.1.1. General introduction of microglia

Microglia are a type of glial cell and has the feature of myeloid lineage cells in the CNS. In humans and other vertebrates (Peters, Josephson et al. 1991; Long, Kalehua et al. 1998), represent 5–20% of all glial cells (Pelvig, Pakkenberg et al. 2008; Lyck, Santamaria et al. 2009). They are key regulators in normal and injured brain (Kettenmann, Hanisch et al.; Kreutzberg 1996; Hanisch and Kettenmann 2007; Ransohoff and Perry 2009). In the healthy brain, microglia are highly heterogeneous and exhibit a resting phenotype characterized morphologically by extensively ramified processes. The “resting” microglia are extremely dynamic, constantly surveillance and changing their morphology by extending and retracting motile processes (Davalos, Grutzendler et al. 2005; Nimmerjahn, Kirchhoff et al. 2005), in order to maintain the normal tissue homeostasis. Studies have also suggested that they are also involved in normal brain development (Tremblay, Stevens et al. 2011) by secreting neurotrophic factors, such as insulin like growth factor 1 (IGF1), brain derived neurotrophic factor (BDNF), transforming growth factor- $\beta$  (TGF $\beta$ ) and nerve growth factor (NGF). In addition, phagocytic functions of microglia have been suggested to support neurogenesis and synaptogenesis (Madinier, Bertrand et al. 2009). Microglial cells rapidly respond to pathogens and traumatic stimuli and transform to an ‘amoeboid’ activated phenotype. The

stages of microglial activation were also defined based on morphological, molecular, and functional characteristics, with fully activated microglia presenting themselves like other peripheral macrophages. Once activated, microglia produce many pro-inflammatory mediators which contribute to the clearance of pathogen infections. However, although an efficient immune response is required for the CNS defense against invading pathogens, excessive microglia activation may lead to tissue injury and pathological damage to the CNS such as neurodegeneration. As a first line of defending cells in the immune privileged CNS, a crucial function of microglia is to generate significant innate and adaptive immune responses (Fig 1.2). Innate immunity is the initial antigen-nonspecific response that results in the microglial phagocytic response and rapid production of chemokines and inflammatory cytokines. By presenting the antigen and interaction with T cells, further adaptive immunity can be induced by microglia (Schwartz, Butovsky et al. 2006).



**Fig 1.2. Activated microglia participate in both innate and adaptive immune responses.**

Microglia can respond to various pathogen-associated molecular patterns found on bacteria (e.g. LPS), viruses or aggregated  $\beta$ -amyloid ( $A\beta$ ). Following activation, activated microglia differentiate into phagocytes and produce various kinds of antimicrobial peptides, cytokines (such as TNF and IL1 $\beta$ ), chemokines (such as CCL2), reactive oxygen species (ROS) and nitric oxide (NO). These molecules have key roles in innate immunity and are characteristic features of the classical M1-like microglial cell phenotype. On the other hand, activated microglia also upregulate the expression of MHC class II molecules to enable them to become antigen-presenting cells (APCs) and present antigens to T cells through the T cell receptor (TCR). In addition, activated microglia produce pro-inflammatory cytokines (such as IL-12) to skew CD4<sup>+</sup> T cells into Th1 cells (which produces IFN $\gamma$ ), IL-23, and TGF $\beta$  to differentiate and activate Th2 cells (which produces IL4). Those factors participate in paracrine and autocrine interactions with the microglia. Both IFN $\gamma$  and IL-4 can induce microglia to express MHC-II and hence to function as APCs. Therefore, classically activated microglia contribute to both innate and adaptive immunity. Images modified from (Saijo and Glass 2011).

## 1.2. Gliomas

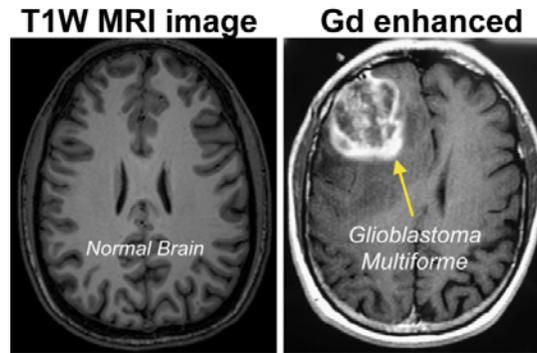
### 1.2.1. Primary brain tumor-gliomas

Gliomas are the most common primary brain tumors. There are nearly 100 different types of intrinsic brain tumors in the CNS (<http://cancerhelp.cancerresearchuk.org/>). Gliomas are graded on a world health organization (WHO) derived scale of I to IV according to their degree of malignancy as characterized by various histological features accompanied by genetic alterations (Furnari, Fenton et al. 2007) (see Fig 1.3).

|                         | <b>Pilocytic Astrocytoma (WHO grade I)</b>                 | <b>Diffuse Astrocytoma (WHO grade II)</b>              | <b>Anaplastic Astrocytoma (WHO grade III)</b> | <b>Glioblastoma a.k.a. Glioblastoma Multiforme (WHO grade IV)</b> |
|-------------------------|--|--|---|---|
| <b>Age of Onset</b>     | First two decades of life                                  | 30 to 40 yrs   | Early 40s                                     | Mid 50–60s  |
| <b>Typical Location</b> | Throughout the neuraxis. Optic pathway tumors are frequent | Cerebral hemispheres. Pons/brainstem, esp. in children | Cerebral hemispheres                          | Cerebral hemispheres  |
| <b>Average Survival</b> | Years to decades   | Five years   | Two to five years                             | Fourteen months   |

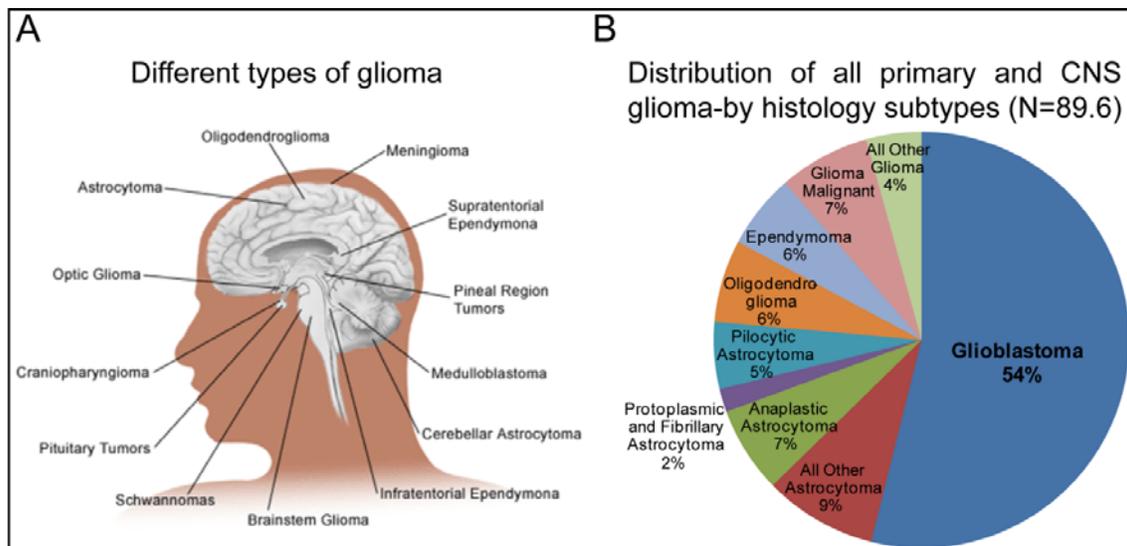
**Fig 1.3. The WHO recognizes 4 major classes of brain tumors.** Of these, 60% belong to one major class—the astrocytic tumors, which are classified into four grades in accordance with histopathological appearance and clinical prognosis. As indicated, there is a direct relationship between tumor grade and age of onset and an inverse relationship between tumor grade and time to death. A subset of grade IV gliomas are thought to arise via progression of diffuse astrocytomas (WHO grade II) or anaplastic astrocytomas (WHO grade III). Image copy from (Stiles and Rowitch 2008)

In particular, the grade IV, glioblastoma multiforme (GBM), is the most common (see Fig 1.5) and aggressive (see Fig 1.4) brain tumor of adults, and is characterized by diffuse infiltration throughout the brain parenchyma, robust angiogenesis, resistance to apoptosis, thrombosis, necrosis, and genomic instability. "Multiforme" derives from the histopathologic features of the tumor's varied morphologically and the presence of heterogeneous cell populations within a tumor. In this study, we focus on tumors of the astrocytic series, emphasizing grade IV GBM (also called high grade gliomas).



**Fig 1.4. T1-weighted MRI images illustrate the aggressiveness of GBM.** (Images modified from <http://www.tumblr.com/tagged/contrast+agent>)

Gliomas are named based on the majority of the cell types are found and/or developed from (see Fig 1.5\_A). For example, gliomas can be classified histologically and immunohistochemically as astrocytomas (astrocytes), ependymoma (ependymal cells), oligodendrogliomas (oligodendrocytes), or mixture of the other types e.g., oligoastrocytomas. Most of the brain tumors are also named according to the brain area in which they begin or are growing in (Fig 1.5\_A). As GBM is the most common primary CNS tumor in the USA and European countries, each year newly diagnosed patients with GBM accounting about 54% of all gliomas (Fig 1.5\_B).



**Fig 1.5. Brain tumors characterizations. A,** Gliomas are categorized by the type of cell where the tumor begins, or they are also categorized by the area of the brain where they occur. The most common types of brain tumors are gliomas. **B,** Distribution of all primary CNS gliomas.

(images modified from <http://www.saintjosephsgammaknife.org/brain-tumors-types> and Central Brain Tumor Registry of the United States report)

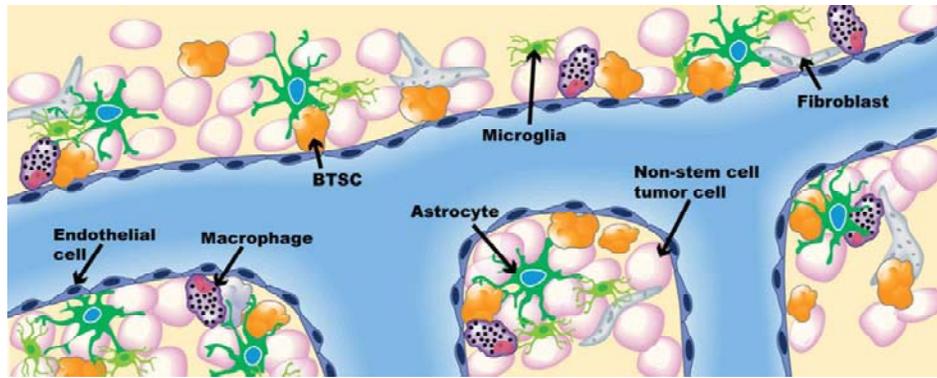
### **1.2.2. Epidemiology, etiology and treatment**

The overall incidence rate of gliomas was 5.05 per 100,000 person–years (The Central Brain Tumor Registry of the United States; CBTRUS). Malignant gliomas may manifest at any age including congenital and childhood cases. Peak incidence is, however, in adults older than 40 years. Males are more frequently affected than females. By far, little is known about the etiology of primary brain tumors. There are two factors so far been concluded to affect glioma risk: environmental factors and inherited genetic mutations. Environmental factors such as food, ionic radiation, or chemicals may cause changes to genes. In addition, some preliminary evidence have shown people with allergic conditions and high levels of serum IgE can also be at glioma risk (Brenner, Butler et al. 2007)

The treatment and prognosis depend upon the tumor grade. Surgery is the most important treatment for most gliomas. The current standard of care for newly diagnosed GBM is surgical resection with concomitant daily chemotherapy e.g. temozolomide (TMZ) and radiotherapy (Wick, Weller et al. 2011). Even after treatment, the median survival rate of GBM patients is only 12 to 15 months (Wen and Kesari 2008).

### **1.2.3. Cellular heterogeneity of glioma microenvironment**

As shown in the glioma tissue obtained by surgical resection, high grade (HG) glioma does not only contain tumor cells but also a massive amount of non transformed cells (e.g. stromal cells) that either infiltrate the tumor, attracted by tumor-secreted molecules such as cytokines, chemokines, and growth factors, or that are engulfed during the tumor's uncontrolled growth (Zhai, Heppner et al. 2011). In gliomas, the microenvironment is composed by microglia, macrophages, astrocytes, brain tumor stem cells (BTSC), neural progenitors, oligodendrocytes, fibroblasts, pericytes, and endothelial cells (see Fig 1.6). Recent studies suggest that brain tumor BTSC contain the capacity for sustained self-renewal and tumor propagation by means of contribute to therapeutic resistance and tumor angiogenesis (Li, Wang et al. 2009). Stromal cells in the glioma tumor compartment are influenced by the tumor, and vice versa.



**Fig 1.6. The glioblastoma (GBM) microenvironment.** It consists of several stromal cell types which are believed to make distinct contributions to tumor progression and invasion. These cells include but are not limited to astrocytes, macrophages, pericytes, fibroblasts, and endothelial cells. Image copy from (Zhai, Heppner et al. 2011).

### 1.2.4. Cell infiltration in glioma

Current evidence supports that glioma cells release factors to attract stromal cells (including: microglia, macrophages, neutrophils, endothelial cells, dendritic cells, fibroblast cells, lymphocytes etc.) to the tumor side (Kessenbrock, Plaks et al. 2010). Accumulation of stromal cells in glioma is due to local production of chemoattractants and growth factors by glioma cells. For example, CCL2 (MCP-1) is produced by glioma cells or astrocytes (Carrillo-de Sauvage, Gomez et al. 2012) and contributes to the entrance of lymphocytes in the brain parenchyma. Also, microglia expressed a specific CCL2 receptor- CCR2. In human glioma, CCL2 has been found to be produced by glioma cells and its expression is positively correlated with microglial infiltration. Moreover, glioma-derived CCL2 acts upon CCR2-bearing microglia, which then produces IL6 to stimulate gliomas (Zhang, Sarkar et al. 2011). Stromal cell-derived factor 1 (SDF-1; CXCL12) has recently been shown to be an important hypoxia induced factor 1 (HIF-1) for the recruitment of bone marrow derived glioma associated cells into GBM (Du, Lu et al. 2008).

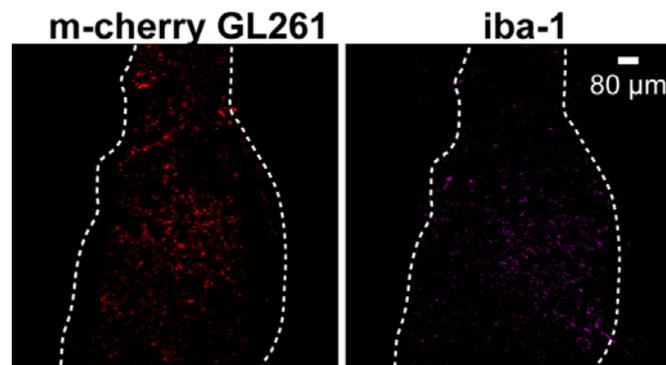
Besides microglia, our group has reported that endogenous neural progenitors from the subventricular zone respond to experimentally-induced tumors by migrating towards, surrounding and infiltrating the primary tumor mass (Glass, Synowitz et al. 2005).

## 1.3. Microglia in glioma

### 1.3.1. Microglia infiltration in glioma

Histopathological studies of glioma tissue have consistently shown high levels of infiltrating microglia and they can amount up to 30% of the tumor mass (Markovic, Glass et al. 2005;

Watters, Schartner et al. 2005). Flow cytometry studies also revealed that one third of the cell populations in glioma expressed microglia markers (Badie and Schartner 2000; Parney, Waldron et al. 2009). There is a positive correlation between the number of microglia and the expression level of MCP-3 (Okada, Saio et al. 2009) or granulocyte/macrophage colony-stimulating factor (GM-CSF) (Komohara, Ohnishi et al. 2008), but a causal relation has not been established. Hepatocyte growth factor (Badie, Schartner et al. 1999) and CCL2 had also been identified as chemo attractant in the glioma context (Leung, Wong et al. 1997; Platten, Kretz et al. 2003).

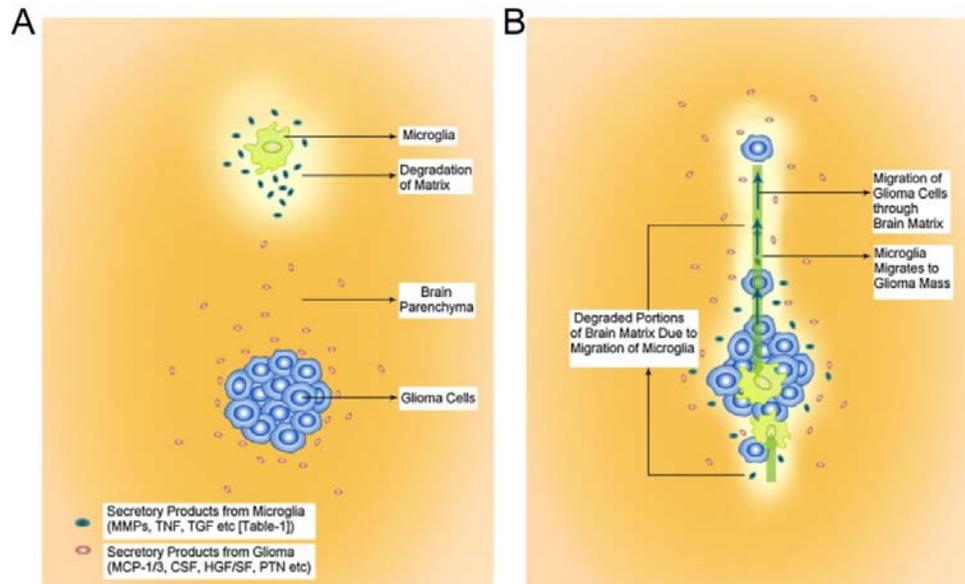


**Fig 1.7. Microglia infiltration in a mouse HG-glioma model.** m-Cherry expressed GL261 glioma cells were injected into mouse brain. After 2 weeks, mice were sacrificed and the brain sections were immunofluorescent stained with Iba-1. Confocal images illustrate that Iba-1 positive microglia were accumulated in the tumor core. Dash lines depict tumor border (image was taken by Min-Chi Ku).

### 1.3.2. Microglia promote glioma progression

Microglia density in HG-gliomas positively correlates with malignancy and invasiveness of HG-gliomas and tumor-associated microglia has an ameboid morphology that is reminiscent of activated microglia in immune reactions. Gliomas have been shown to attract many microglia along with a small population of lymphocytes. The observation that malignant gliomas contain particularly high levels of microglia infiltration links to that microglia may have anti-tumor activity. However, recent evidence have shown that unlike in an inflamed tissue, immune functions in tumor associated microglia are rather suppressed resulting in aberrant MHC Class II expression (Geranmayeh, Scheithauer et al. 2007), loss of phagocytotic activity (Voisin, Bouchaud et al. 2010) impaired anti-inflammatory cytokine secretion (Hussain, Yang et al. 2006) and reduced antigen presenting function (Waziri, 2010; Raychaudhuri et al., 2011). Microglia even contribute to the immunosuppressive environment of gliomas and may promote glioma cell migration and invasion by glioma secreting pro-inflammatory cytokines (Bettinger, Thanos et al. 2002; Schartner, Hagar et al. 2005; Wesolowska, Kwiatkowska et al. 2008). Therefore, microglial cells are not anti tumorigenic,

but rather have been shown to promote glioma growth. Which could be shown by *in vivo* ablation of microglia using the CD11b- HSVTK model in which glioma tumor size was dramatically reduced (Zhai, Heppner et al. 2011). Depletion of microglia attenuate glioma invasion in organotypic brain slices (Markovic, Glass et al. 2005) and results in reduced glioma growth *in vivo* (Markovic, Vinnakota et al. 2009). Altogether, the recent data by us and others indicate that blockade of microglia infiltration into HG-gliomas could be a novel therapeutic target in neuro-oncology.



**Fig 1.8. Microglia favors glioma in metastasis.** **A**, Glioma secretes chemotactic and mitogenic factors causing microglial attraction and activation. Both infiltrated microglia and glioma cells then secrete different matrix degrading enzymes e.g. MMPs and cytokines to promote the invasion. **B**, Eventually glioma cells use the same corridor of dissociated brain tissue matrix for their metastasis that microglia caved to reach the glioma site by degrading the matrix. Image copy from (Ghosh and Chaudhuri 2010).

#### 1.4. Astrocytes in glioma

Astrocytes have traditionally been considered as supporting cells in the CNS. Indeed, astrocytes are the most abundant glia cell population in the mammalian brain. Interest in astrocyte function has increased dramatically in recent years because of their newly discovered functions in forming communication pathway for neuronal plasticity (Anderson and Nedergaard 2003) and participation in the local innate immune response triggered by a variety of insults (Farina, Aloisi et al. 2007). When CNS responds to diverse neurologic injuries it is accompanied with a vigorous activation of astrocytes. The increase in the number and size of cells expressing glial fibrillary acidic protein (GFAP), is a phenomenon generally referred to reactive astrocytosis (Eddleston and Mucke 1993). Earlier studies have shown

that reactive astrocytes are also found in the glioma environment and are associated with increased glioblastoma invasion (Edwards, Woolard et al. 2011). Thus, brain tumor related lesion and subsequent inflammation in general trigger reactive astrocytosis. Those brain tumor associated activated astrocytes secrete chemotactic factors (e.g. SDF1/CXCL12) which might support glioma invasion. Reactive astrocytes expressing sonic-hedgehog are highly concentrated at the perivascular region of HG-gliomas and their density is associated with an increasing grade in PDGF-induced gliomas (Becher, Hambardzumyan et al. 2008). Astrocytes may contribute to glioma growth, by mediating the release of TGF- $\beta$  (Dhandapani, Khan et al. 2007), TGF- $\alpha$  (Sharif, Legendre et al. 2007) and pro-MMP-2 (Le, Besson et al. 2003), and the coupled astrocytic network (connexins and tight junction) facilitates glioma cell invasion (Lin, Takano et al. 2002). In addition, the capacity for astrocytes to produce neurotrophic factors that function in tumor cell invasion has implicated them in the promotion of glioma growth (Hoelzinger, Demuth et al. 2007).

### **1.5. Neural progenitor cells in glioma**

In adult brain, neural progenitor cells (NPC) are endogenous cells in SVZ and SGZ which can continuously self-renew and have the potential to generate intermediate and mature cells of both glial and neuronal lineages (Doetsch 2003). Emerging evidence have shown that endogenous NPC are attracted to various brain lesions, such as brain tumors and areas of neurodegeneration. The attraction of NPC to experimental glioblastoma was initially observed with exogenously cultivated and immortalized precursors (Aboody, Brown et al. 2000). In addition, large numbers of endogenous NPC migrate towards experimental brain tumors and eliminated tumor growth (Markovic, Glass et al. 2005; Walzlein, Synowitz et al. 2008). Various factors are involved in the NPC attraction such as chemokines and growth factors (Belmadani, Tran et al. 2006). It has been found that NPC home into pathologic brain tissue and possibly also to tumors due to expression of CXCR4 (Fasano, Phoenix et al. 2009).

Recent studies have shown that NPC potentially have an anti-tumor effect. In vitro cultured NPC release soluble factors that can cause glioblastoma cell death and perform an anti-tumor response by BMP7 (Chirasani, Sternjak et al. 2010). In mouse glioblastoma models, using syngenic implantation of glioblastoma cells, it was shown that attracted NPC correlate with improved survival (Markovic, Glass et al. 2005; Walzlein, Synowitz et al. 2008).

### **1.6. Nerve/glial antigen 2 (NG2) cells in glioma**

NG2 is a chondroitin sulfate proteoglycan expressed by a subpopulation of glial cells called NG2 cells in the developing and mature CNS. In the developing and adult CNS, up to 5–10%

glia expressed NG2 (Trotter, Karram et al. 2010). Not only glial cells, pericytes lining blood vessels can also express NG2 (Ozerdem, Grako et al. 2001). During development and normal adulthood, NG2 expressing glia generate oligodendrocytes (Richardson, Young et al. 2011). In many types of injury and pathological situations (including tumors), NG2 protein is up-regulated and known for its important role in cell proliferation, migration, and angiogenesis. In addition, NG2 expressing cells respond to injury by increasing proliferation, up-regulation of NG2 expression and generating remyelinating oligodendrocytes and possibly astrocytes when required (Diers-Fenger, Kirchhoff et al. 2001). Recent study showed that some population of glioma cells co-expressed NG2 in GBM (Al-Mayhany, Grenfell et al. 2011). Overall, NG2 may contribute to critical processes such as cell proliferation, glioma vasculature, cell motility and cell survival in glioma. Moreover, several reports have correlated the expression of NG2 proteoglycan with the degree of malignancy of the glioma (Stallcup and Huang 2008); however, the detail role of NG2 in glioma is still not known.

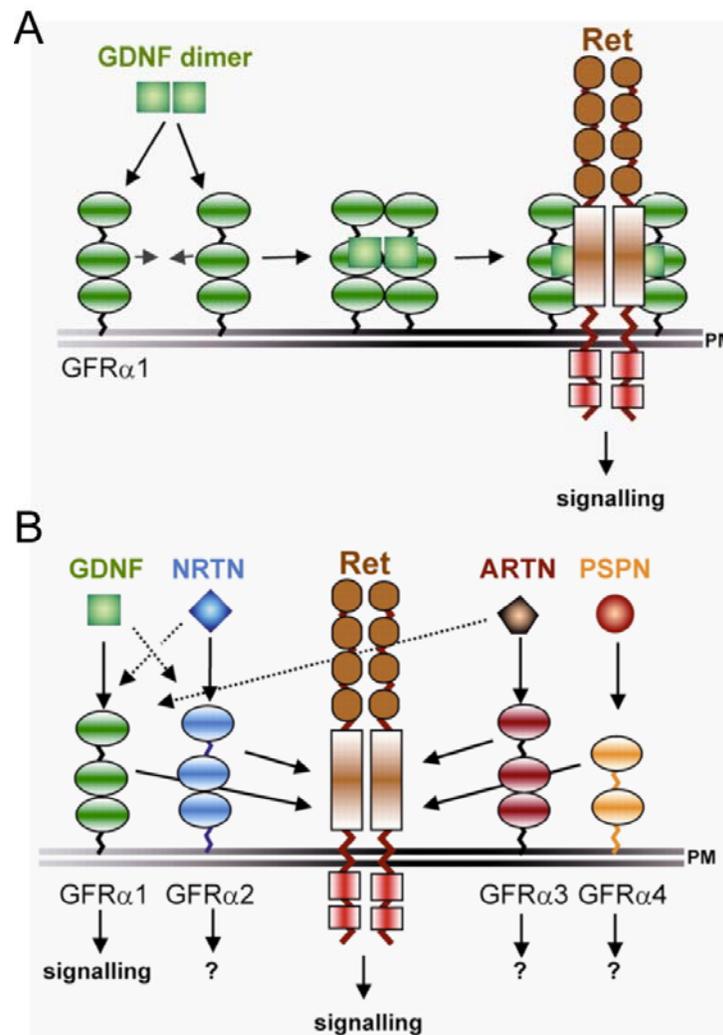
## **1.7. GDNF in CNS**

### **1.7.1. Function of GDNF**

GDNF is a potent trophic factor for a variety of neuronal cell populations in the CNS. It was first discovered in the conditioned medium of the B49 rat glioma cell line (Lin, Doherty et al. 1993) and since then, three other GDNF related proteins (artemin, neurturin and persephin) are identified as members of the GDNF family. GDNF family proteins are distant members of the TGF $\beta$  superfamily. The physiological function of GDNF are cell differentiation (while development), promote dopaminergic neuron cell survival and neurite outgrowth, synaptic plasticity and cell proliferation. In addition to its neurotrophic activity, GDNF has important roles outside of the nervous system (Sariola and Saarma 2003). Among other functions, GDNF acts as a morphogen in kidney development, as a migration factor for neural crest cells, and as a regulator of the differentiation of spermatogonia (Airaksinen and Saarma 2002). Artemin promotes the survival and growth of various peripheral and central neurons, including sympathetic and dopaminergic neurons. Neurturin exerts a positive effect on the survival of a variety of neurons and possibly other cell types. Persephin promotes the survival and growth of central dopaminergic and motor neurons, and is also involved in kidney development. Four GDNF family receptors (GFRs) have been identified, which determine the ligand specificity. GDNF ligands bind to preference GFRs e.g. GFR $\alpha$ -1 binds preferentially to GDNF, GFR $\alpha$ -2 to neurturin, GFR $\alpha$ -3 to artemin, and GFR $\alpha$ -4 to persephin. *In vitro* studies have shown that following GDNF binding to GFR $\alpha$ -1 the resulting complex recruits Ret receptor tyrosine kinase, leading to its activation by dimerization and

autophosphorylation at specific cytoplasmic tyrosine residues, thus initiating a number of downstream intracellular pathways (Trupp, Raynoschek et al. 1998). RET is activated by a complex of a GDNF ligand (GFL) and a GFR. GFR $\alpha$  proteins are attached to the plasma membrane through a GPI-anchor and consist of three (GFR $\alpha$ 4 has only two) globular cysteine-rich domains joined together by adapter sequences. It is important to know that amount those GDNF ligands and receptors, deletion of GDNF, GFR $\alpha$ -1, and RET in mouse is lethal at birth. Deletion of GFR $\alpha$ -2 mice grow poorly after weaning (Airaksinen and Saarma 2002).

Recent studies focus on factors that regulate the cross-talk between glioma cells and glial cells. There are a number of candidate factors, which appear to mediate the interaction between glioma cells and microglia or other intrinsic brain cells. However, the role of glial cell derived neurotrophic factor (GDNF) in this respect has never been discussed.



**Fig 1.9. GDNF-family ligand and their receptors.** **A**, GFR proteins are attached to the plasma membrane by a glycosyl phosphatidylinositol (GPI) anchor. Homodimeric GDNF first binds to two molecules of GFR $\alpha$ 1. The complex dimerizes the two molecules of Ret. **B**, All GDNF ligands activate

Ret tyrosine kinase via different GFR $\alpha$  receptors. Solid arrows indicate the preferred functional ligand-receptor interactions, whereas dotted arrows indicate possible crosstalk. Images copy from (Sariola and Saarma 2003)

### 1.7.2. GDNF in glioma

GDNF is not only a trophic factor, but also a chemoattractant for various types of cells (Wan and Too; Cornejo, Nambi et al.; Dudanova, Gatto et al. 2010; Koelsch, Feng et al. 2010; Lu, Leung et al.; Wan and Too 2010). The first report described GDNF stimulate epithelial cell migration and chemoattraction (Tang, Worley et al. 1998). In recent years it had been reported as chemoattractant for carcinoma cells (Iwahashi, Nagasaka et al. 2002), Schwann cells (Cornejo, Nambi et al.), neuronal progenitor cells (Paratcha, Ibanez et al. 2006), breast cancer cells (Esseghir, Todd et al. 2007), pancreatic cancer cell (Gil, Cavel et al. 2010), and glioma cells (Wan and Too 2010). Although GDNF has been studied for several years, the role of this potent chemoattractant factor in the crosstalk between glioma and microglia has not been investigated.

Importantly, GDNF has been found highly expressed in human HG-gliomas compared to normal brain (Wiesenhofer, Stockhammer et al. 2000). *In vitro* studies showed C6 glioma cells that gain of function of GFR $\alpha$ -1 and GDNF resulted in increased cell migration compared to unstimulated cells, whereas a low-grade glioma cell line that expressed lower amounts of GFR $\alpha$ -1 reacted to stimulation with a modest increase in GDNF-dependent cell motility (Song and Moon 2006). Application of GDNF on cultured glioma cells promotes the survival, proliferation and activation of glioma cells (Lu, Leung et al. 2010). Therefore, GDNF, which is nearly universally expressed throughout the brain, could foster the maintenance of glioma cell invasion through its receptors that are expressed on invasive glioma cells.

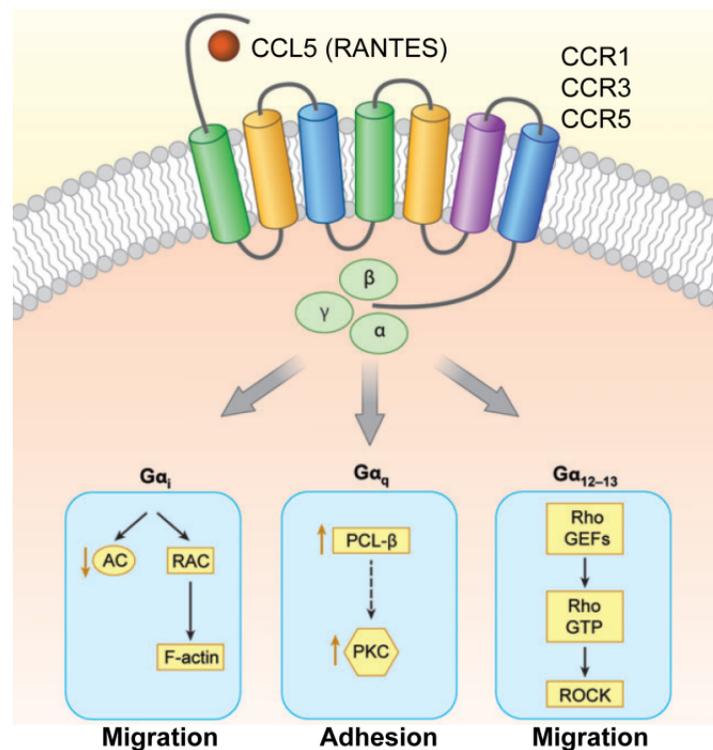
### 1.7.3. GDNF and microglia

Several studies have shown that activated microglia under pathological conditions increased GDNF production in the injured striatum or spinal cord suggesting that endogenous GDNF may provide neuronal protection after the CNS injury (Soler, Dolcet et al. 1999; Satake, Matsuyama et al. 2000; Boscia, Esposito et al. 2009). In the CNS inflammation, LPS enhances synthesis of GDNF in cultured rat microglia (Miwa, Furukawa et al. 1997) and in the *in vitro* ischemia model it appeared to induce the expression of GDNF in microglia (Lee, Lin et al. 2004). Furthermore, *in vitro* cultured rat primary microglia expressed GDNF and GFR $\alpha$ -1 receptor (Honda, Nakajima et al. 1999). Not only producing GDNF, upon GDNF stimulation, microglia increased the nitric oxide (NO) production (Chang, Fang et al. 2006) and GDNF supported microglia survival (Salimi, Moser et al. 2003).

## 1.8. Chemokines in CNS

### 1.8.1. Introduction of chemokine system

Chemokines are a group of small soluble factors (7–14 kDa) that promote directional migration of leukocytes. There are approximately 50 chemokines and 20 chemokine receptors identified to date are classified into CXC, CC, CX3C or C chemokines based on the positioning of the conserved cysteine residues. Chemokines exert their chemotactic functions by binding to chemokine receptors, which conserve seven-transmembrane-domain and coupled to heterotrimeric G protein, also called G protein-coupled receptors (GPCRs). With only few exceptions, most chemokines act on more than one receptor. Depending on their function, chemokines can be homeostatic, inflammatory, or both. Homeostatic chemokines are constitutively expressed and are important for maintaining physiological processes such as growth-regulatory properties, while the expression of inflammatory chemokines are induced by inflammatory stimuli (Murdoch and Finn 2000). Chemokines mediate the host defense mechanisms through development and maintenance of innate and adaptive immunity. In CNS, following CNS damage due to disease or injury, chemokines can attract peripheral or intrinsic cells like NPC to the site (Leong and Turnley 2011). A new concept emerging from several recent studies showed that chemokines are not only inflammatory mediators in the brain but also act as potential modulators in neurotransmission (Rostene, Guyon et al. 2010).



**Fig 1.10. Chemokines and receptor signaling pathway.** Chemokine receptors are seven-transmembrane molecules coupled to heterotrimeric G proteins. Chemokine (example here is CCL5) binds to chemokine receptors (example here is CCL5 receptors) then the  $\beta$ - and  $\gamma$ -subunits are assembled into  $\beta\gamma$  dimers that act as functional units. The  $\alpha$ -subunits bind guanine nucleotides, being active when GTP is bound. The G proteins are usually classified by the nature of their  $\alpha$ -subunit— $\alpha_i$ ,  $\alpha_q$ , and  $\alpha_{12/13}$ . Chemokine receptors can signal through different G $\alpha$ -protein families, leading to distinct transduction pathways and biological effects. G $\alpha_i$  coupling regulates gradient sensing and F-actin polymerization at the leading edge of a migrating cell. Image modified (Viola and Luster 2008).

### 1.8.2. Chemokines in glioma

Chemokines were described originally in the context of providing migration cues for leukocytes. They are now known to have broader activities, including those that favor tumor growth, angiogenesis and metastasis (Belperio, Keane et al. 2000). Interestingly, the chemokine network also contributes to the progression of gliomas, mainly by intensifying their characteristic invasive character (Domanska, Kruizinga et al. 2010).

Several chemokines have now been described in glioma cells *in situ* and *in vitro*, and these including CCL2 (MCP-1) (Leung, Wong et al. 1997), CXCL8 (IL8) (de la Iglesia, Konopka et al. 2008), CCL5 (RANTES) (Kouno, Nagai et al. 2004), CXCL12 (SDF-1) (Ehtesham, Winston et al. 2006) and receptor CX3CR1 (Locatelli, Boiocchi et al. 2010). The function of these chemokines in glioma biology remains uncertain, but they might account for the increased malignancy of glioma cells. For example, a role for chemokines in regulating angiogenesis is suggested by the observation that hypoxic/anoxic insults to glioma cells *in vitro* induced an increase in IL8 mRNA. Also, SDF-1 and CXCR4 are co-localized with regions of angiogenesis in glioma specimens *in situ*. A majority of human glioma lines expressed a common chemokine receptor, CXCR4. SDF-1 acts on CXCR4 to regulate glioma survival, and this adds to the potential roles of chemokines in the CNS (Zhou, Luo et al. 2002). Up-regulation of the CCL3L1 (can binds to chemokine binding protein 2 and CCR5), CCR3 and CCR5 chemokine-receptors system is involved in the progression of glioblastoma (Kouno, Nagai et al. 2004).

### 1.8.3. Chemokine CCL5 and its receptors in glioma

CCR5 has generated widespread interest because of its role as a co-receptor for HIV and multiple sclerosis (Teixeira, Vilela et al. 2010). However, to date there are only a limited number of *in vitro* studies that implied the importance of CCL5 and glioma. Glioma cells as well as primary astrocytes produced CCL5 upon incubation with TNF $\alpha$  or IL1 (Barnes, Huston et al. 1996). Retinoic acid poly IC induced CCL5 expression in human glioblastoma cell line U373 (Yoshida, Imaizumi et al. 2007). Glioma cell line co-culture (Ehtesham,

Winston et al. 2006) with human THP-1-derived macrophages increased expression of the CCL3L1, CCR3 and CCL5 proteins (Hong, Teng et al. 2009). Those data indicated that CCR5 chemokine-receptors system is involved in brain tumorigenesis, especially in the progression of glioblastoma (Kouno, Nagai et al. 2004).

#### **1.8.4. CCL5 and its receptors in microglia**

CCL5 is known as a migration factors for microglia upon inflammatory stimulation. Study has shown that CCL5 and CCL2 are prominent chemokines that mediate the chemotaxis of microglia toward beta-amyloid (A $\beta$ ) aggregates (Schlachetzki, Fiebich et al. 2010). Broadprofiles of chemokine receptors expressed on resting and activated adult human microglial cells has been studied. For instance, microglia express highest levels of CXCR1, CXCR3 and CCR3. They also expressed CCR4, CCR5, CCR6, CXCR2, CXCR4 and CXCR5 at lower levels. Activation of microglia with the inflammatory cytokine TNF $\alpha$  and IFN $\gamma$  increased the expression of some but not all (Flynn, Maru et al. 2003).

The chemokine receptors CCR1 (CCL5 receptor), CCR2 and CCR5 (CCL5 receptor) have been found to recruitment of both infiltrating macrophages and resident microglia to sites of CNS inflammation. In addition, CCR1 (CCL5 receptor), CCR2, CCR3 (CCL5 receptor), CCR5 (CCL5 receptor), and CXCR2 have been found to be expressed on microglia in many neurodegenerative diseases, such as multiple sclerosis and Alzheimer's disease (Skuljec, Sun et al. 2011). Furthermore, CCR1 is expressed by microglia in neurofibrillary tangle-bearing neuritic processes (Ab42 plaques) in human brain (Halks-Miller, Schroeder et al. 2003). The activation of CCR5 is link to Ca<sup>2+</sup> increases in human microglia (Shideman, Hu et al. 2006). During CNS lesions (such as in multiple sclerosis), activated microglia upregulate CCR5 expression (Trebst, Sorensen et al. 2001).

### **1.9. Hollow fiber (HF)**

#### **1.9.1. General application of HF**

The principle of cell encapsulation is aim to entrap viable cells within the confines of semi-permeable (porous) membranes which allows free exchange of nutrients, oxygen, and substances. It allows the *in situ* or *in vivo* delivery of secreted proteins to treat different pathological conditions and avoid the immunological interaction with host cells. Encapsulation can be classified into two categories: microcapsules and macrocapsules. Microcapsules are characterized by dimensions of the order of hundreds of microns or less. The small encapsulation volume enables capsule implantation in microvasculature, deep

tissue and difficult to access sites. Macrocapsules are characterized by dimensions of the order of 0.5–1.5 mm in diameter and a few cm in length. The large encapsulation volume of macrocapsules allows higher cell loading densities (Krishnamurthy and Gimi 2011). The initial experiments with cell encapsulation were initiated in the 1930s, but serious interest in such technology developed in the 1970s with the encapsulation of pancreatic tissue into synthetic membranes (Chick, Perna et al. 1977). 30 years later now it has been used in numerous conditions such as *in vivo* noninvasive imaging of molecular pathways in cells within hollow fibers (Zhang, Chen et al. 2008).

Applications of encapsulated cell technology apart from cell transplantation

|   |
|---|
| Large-scale production of cell-derived molecules in biotechnology industry<br>Clonal selection of desired cell phenotypes<br>In vitro culture of cells dependent on close cell–cell contact<br>In vivo cell culture<br>Reproductive technology<br>Cytotoxicity testing<br>Cell therapy: Local delivery of therapeutic factors |
|---|

**Table 1.1 List of applications of hollow fiber method.** Table modified from (Uludag, De Vos et al. 2000)

### 1.9.2. HF for studying soluble factors in the CNS

During the last decades, there has been significant progress in the development of HF cell encapsulation and achieved drugs or gene deliver for treatment of neurological disorders and tumors in the brain (Zhong and Bellamkonda 2008). Recent studies have shown that encapsulated dopamine producing cells and cells delivering neurotrophic factors might be a transplantation model for treating Parkinson's disease (Li, Williams et al. 1999; Kim, Hitchcock et al. 2005; Ye, Zhao et al. 2009). Furthermore, the endostatin releasing cells from the capsules led to an induction of apoptosis and hypoxia within treated brain tumors (Read, Sorensen et al. 2001).

## 2. Aim of the study

High grade (HG) gliomas are the most malignant brain tumors. The glioma microenvironment comprises numerous cell partners, signaling molecules and pathways that influence the hallmark of HG glioma. Despite the finding that brain resident cells are the main characters in either recovery or tumor invasiveness, little is known about the mechanisms and role of brain resident cells and glioma cell interaction. In this thesis I would like to investigate how brain intrinsic cells interact with glioma cells. Following questions were raised:

1. What types of cell in the CNS participate in glioma formation?
2. What are the factors involved in glioma-intrinsic cells crosstalk?
3. What is the outcome when interfere with glioma-intrinsic CNS cell interaction?

The impact of glioma released factors along with the relationship between glioma and intrinsic brain cells are planed to achieve by using hollow fiber (HF) cell encapsulation method. By both tumor cell inoculation method and HF method, the cell network in glioma can be observed. Further applying gene knockdown techniques, the impact of soluble factor for glioma pathology can be characterized.

### 3. Materials and Methods

#### 3.1. Materials

##### 3.1.1. Cell culture media and supplements

3.1.1.1. Table 3.1 Cell culture media and supplements

| Product Name                                | Supplier                       |
|---|--------------------------------|
| Dulbecco's phosphate-buffered saline (DPBS) | Invitrogen, Darmstadt, Germany |
| Dulbecco's Modified Eagle Medium (DMEM)     | Invitrogen, Darmstadt, Germany |
| DMSO  | Sigma-Aldrich, Munich, Germany |
| EGF   | Cell Systems, Washington, USA  |
| Fetal calf serum (FCS)                      | Invitrogen, Darmstadt, Germany |
| FGF   | Cell Systems, Washington, USA  |
| L-Glutamine                                 | Invitrogen, Darmstadt, Germany |
| Penicillin/Streptomycin                     | Invitrogen, Darmstadt, Germany |
| RPMI 1640                                   | Invitrogen, Darmstadt, Germany |
| Trypsin-EDTA                                | Invitrogen, Darmstadt, Germany |

##### 3.1.2. Drugs and chemicals

3.1.2.1. Table 3.2 Drugs and chemicals

| Product Name                            | Supplier                                     |
|---|--|
| 4',6-Diamidin-2-phenylindol (DAPI)      | Sigma-Aldrich, Munich, Germany               |
| Alamar blue                             | Invitrogen, Darmstadt, Germany               |
| Ammonium persulphate (APS)              | Merck, Darmstadt, Germany                    |
| Agarose                                 | Lonza, Cologne, Germany                      |
| Acrylamide/Bisacrylamide 30 % solution  | Sigma-Aldrich, Munich, Germany               |
| Aqua Poly mount                         | Polysciences Europe GmbH, Eppelheim, Germany |
| Bromophenol blue                        | Sigma-Aldrich, Munich, Germany               |
| Bovine serum albumine (BSA)             | Carl Roth GmbH, Karlsruhe, Germany           |
| $\beta$ -mercaptoethanol                | Merck, Darmstadt, Germany                    |
| Dimethylsulphoxide (DMSO)               | Sigma-Aldrich, Munich, Germany               |
| Dharmafect 4                            | Dharmacon, Chicago, USA                      |
| Ethidium bromide                        | Carl Roth GmbH, Karlsruhe, Germany           |
| G418 (neomycin)                         | Sigma-Aldrich, Munich, Germany               |
| Glycerol                                | Sigma-Aldrich, Munich, Germany               |
| Isofluorane                             | Minrad International, New York, USA          |
| Isopentane                              | Carl Roth GmbH, Karlsruhe, Germany           |
| Ketamin 10%                             | WDT, Garbsen, Germany                        |
| Lipofectamine 2000 transfection reagent | Invitrogen, Darmstadt, Germany               |

|   |   |
|---|---|
| Lipofectamine LTX transfection reagent        | Invitrogen, Darmstadt, Germany            |
| Low melting point agarose                     | Promega, Wisconsin, USA                   |
| Methanol                                      | Carl Roth GmbH, Karlsruhe, Germany        |
| N,N,N',N' Tetramethyl-Ethylen-Diamine (TEMED) | Amresco, Ohio, USA                        |
| Normal donkey serum (NGS)                     | Sigma-Aldrich, Munich, Germany            |
| Pierce ECL Western Blotting Substrate         | Thermo Fisher Scientific, Rockford, USA   |
| Ponceau S solution                            | Sigma-Aldrich, Munich, Germany            |
| Paraformaldehyde                              | Merck, Darmstadt, Germany                 |
| Protease inhibitor cocktail                   | Roche, Berlin, Germany                    |
| Phusion polymerase                            | FINNZYMES OY, Keilaranta, Finland         |
| RIPA buffer                                   | Sigma-Aldrich, Munich, Germany            |
| Puromycin                                     | Sigma-Aldrich, Munich, Germany            |
| Rompun  | Bayer HealthCare AG, Lerverküsen, Germany |
| Rhodamine Phalloidin                          | Tebu-Bio, Offenbach, Germany              |
| Scrambled non-targeted siRNA                  | Dharmacon, Chicago, USA                   |
| shGDNF (OmicsLink™ shRNA clones)              | GeneCopoeia, Maryland, USA                |
| siRNA GDNF (on target plus SMARTpool)         | Dharmacon, Chicago, USA                   |
| siRNA CCL5 (on target plus SMARTpool)         | Dharmacon, Chicago, USA                   |
| siRNA GAPDH (SMARTpool)                       | Dharmacon, Chicago, USA                   |
| Sodium dodecylsulfate polyacrylamide (SDS)    | Sigma-Aldrich, Munich, Germany            |
| Sucrose                                       | Merck, Darmstadt, Germany                 |
| SuperScript II reverse transcriptase          | Invitrogen, Darmstadt, Germany            |
| Tissue Tek® Compound (OCT)                    | Weckert Labortechnik, Kitzingen, Germany  |
| Tris-base                                     | Carl Roth GmbH, Karlsruhe, Germany        |
| Tris-HCl                                      | Carl Roth GmbH, Karlsruhe, Germany        |
| Triton X-100                                  | Carl Roth GmbH, Karlsruhe, Germany        |
| Trypan blue                                   | Sigma-Aldrich, Munich, Germany            |
| Tween 20                                      | Merck, Hohenbrunn, Germany                |

### 3.1.3. Cytokines and commercial Kits

#### 3.1.3.1. Table 3.3 Recombinant proteins

| Product Name                     | Supplier                           |
|----------------------------------|------------------------------------|
| Recombinant murine GDNF          | Peprtech Germany, Hamburg, Germany |
| Recombinant murine CCL5 (RANTES) | Peprtech Germany, Hamburg, Germany |

#### 3.1.3.2. Table 3.4 Commercial Kits

| Product Name                         | Supplier                                |
|--------------------------------------|---|
| Cell proliferation ELISA, BrdU       | Roche, Berlin, Germany                  |
| GDNF Emax® Immunoassay System        | Promega, Wisconsin, USA                 |
| InviTrap Spin Universal RNA Mini Kit | Invitek, Berlin, Germany                |
| Micro BCA Protein Assay Kit          | Thermo Fisher Scientific, Rockford, USA |
| PCR Kit                              | Takara, Shiga, Japan                    |
| CCL5 (RANTES) ELISA mini Kit         | Peprtech, Hamburg, Germany              |

|                                 |                             |
|---------------------------------|-----------------------------|
| RNeasy Kit                      | Qiagen, Düsseldorf, Germany |
| Qiagen® Hi-Speed® Maxiprep kit® | Qiagen, Düsseldorf, Germany |

### 3.1.4. Antibodies

#### 3.1.4.1. Table 3.5 Primary antibodies

| Product Name           | Supplier   |
|------------------------|--|
| Rabbit anti GDNF       | Santa Cruz Biotechnology Inc., Heidelberg, Germany |
| Rabbit anti GDNF       | Abcam, Cambridge, UK                               |
| Rabbit anti Iba-1      | Wako Pure Chemicals, Tokyo, Japan                  |
| Rabbit anti mouse GFAP | Sigma-Aldrich, Steinheim, Germany                  |
| Mouse anti Vimentin    | Abcam, Cambridge, UK                               |
| Mouse anti GFP         | Abcam, Cambridge, UK                               |

#### 3.1.4.2. Table 3.6 Secondary antibodies

| Product Name                                    | Supplier   |
|---|--|
| Alexa Fluor 594-conjugated goat anti-rabbit IgG | Invitrogen, Karlsruhe, Germany                   |
| DyeLight 488-conjugated donkey anti-mouse IgG   | Jackson ImmunoResearch/Dianova, Hamburg, Germany |
| DyeLight 488-conjugated donkey anti-rabbit IgG  | Jackson ImmunoResearch/Dianova, Hamburg, Germany |
| Cy5-conjugated donkey anti-rabbit IgG           | Jackson ImmunoResearch/Dianova, Hamburg, Germany |

### 3.1.5. Lab wares, equipments and devices

#### 3.1.5.1. Table 3.7 Lab wares

| Product Name                              | Supplier  |
|---|---|
| 6, 12, 24, and 96-well Cell Culture Plate | BD Biosciences, Heidelberg, Germany                 |
| 35-mm dish with cover slip                | MatTek Corporation, Massachusetts, USA              |
| 96-well ELISA Plate (NUNC)                | Thermo Fisher Scientific, Rockford, USA             |
| 40 µm cell strainer                       | BD Biosciences, Heidelberg, Germany                 |
| Counting chamber                          | Paul Marienfeld GmbH, Lauda Königshofen, Germany    |
| Hamilton syringe (10 µL, Model 701 SN)    | Hamilton Bonaduz AG, Bonaduz, Switzerland           |
| Hollow fiber                              | Minttech, Minneapolis, USA                          |
| Hybond-P PVDF membrane                    | Amersham Biosciences Europe GmbH, Freiburg, Germany |
| Menzel glass cover slip 24x50 and 24x60mm | Thermo Fisher Scientific, Rockford, USA             |

|   |   |
|---|---|
| Microchemotaxis chamber (Boyden chamber)              | Neuroprobe, Maryland, USA               |
| Parafilm  | Pechiney Plastic Packaging, USA         |
| Pierce Western Blotting Filter Papers                 | Thermo Fisher Scientific, Rockford, USA |
| Polycarbonate membrane (5 $\mu$ m and 8 $\mu$ m pore) | Neuroprobe, Maryland, USA               |
| Stereotactic alignment system                         | David Kopf Instruments, Tujunga, USA    |
| Surgical sewing cone                                  | Johnson & Johnson, New Jersey, USA      |
| Transwell insert (pore size 8 $\mu$ m)                | BD Biosciences, Heidelberg, Germany     |

3.1.5.2. Table 3.8 Devices

| Product Name  | Supplier   |
|---|--|
| 9.4 Tesla small animal MRI system (Biospec 94/20)               | Bruker Biospin, Ettlingen, Germany                 |
| Blotting chamber (Biorad Trans-Blot®SD wet Transfer Cell)       | Biorad, München, Germany                           |
| BioStation IM   | Nikon, Düsseldorf, Germany                         |
| Cryogenically cooled radio-frequency probe (CryoProbe, 400 MHz) | Bruker BioSpin, Ettlingen, Germany                 |
| Cryostat (CM 3050S)   | Leica Microsystems, Wetzlar, Germany               |
| Centrifuges (Eppendorf 5403 and 5417)                           | Eppendorf, Hamburg, Germany                        |
| Eppendorf Thermomixer 5355                                      | Eppendorf, Hamburg, Germany                        |
| Gel electrophoresis chamber                                     | Thermo Fisher Scientific, Rockford, USA            |
| Inverse microscope (Axiovert 25)                                | Carl Zeiss MicroImaging GmbH, Berlin, Germany      |
| Leica confocal microscopy (TCS SP5)                             | Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany |
| Microplate plate reader Infinite M200                           | Tecan, Crailsheim, Germany                         |
| Monitoring and gating system (Model 1025)                       | Small Animal Instruments Inc., New York, USA       |
| PCR machine (T3000 thermocycler)                                | Biometra, Göttingen, Germany                       |
| pH meter  | Hanna Instruments®, Kehl am Rhein, Germany         |
| Stereotactic frame  | David Kopf Instruments, California, USA            |
| Zeiss laser scanning confocal microscope (LSM 710)              | Carl Zeiss MicroImaging GmbH, Berlin, Germany      |

3.1.5.3. Table 3.9 Computer software

| Software                               | Supplier  |
|--|---|
| Adobe Illustrator CS                   | Adobe Systems Inc. USA  |
| Adobe photoshop                        | Adobe Systems Inc. USA  |
| Image J software                       | NIH, Bethesda, USA  |
| Leica LAS AF                           | Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany              |
| MedCalc                                | MedCalc Software, Mariakerke, Belgium                           |
| Mipav software                         | <a href="http://mipav.cit.nih.gov">http://mipav.cit.nih.gov</a> |
| Microsoft Office 2007                  | Microsoft Deutschland, Berlin, Germany                          |
| SPSS software                          | SPSS, Inc., New York, USA                                       |
| Syngene G-Box gel documentation system | Imgen Technologies, Virginia, USA                               |
| Carl Zeiss Zen 2011                    | Carl Zeiss MicroImaging, Berlin, Germany                        |

## 3.2. Methods

### 3.2.1. Cell cultures

#### 3.2.1.1. Mouse cell culture

GL261 murine glioma cells (National Cancer Institute) and non-tumorigenic fibroblast cell-line SCRC-1008 (ATCC) established from C57BL/6 mouse embryos were cultured in DMEM with supplements (200mM glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, and 10% FBS). Primary microglia was prepared from neonatal C57BL/6 mice as previously described (Lyons and Kettenmann 1998; Glass, Synowitz et al. 2005; Markovic, Glass et al. 2005). Briefly, newborn C57BL/6 mice (P0-P3) brains were removed and placed in HBSS. Brain meninges and cerebellum were carefully removed. After washing steps the brains were trypsinized and simultaneously treated with the DNase. The tissue was disintegrated by using a Pasteur pipette and the cell suspension was cultured in poly-L-lysine coated 75T flasks. Cells were cultured in DMEM with supplements and L929 fibroblasts conditioned medium. After one week, microglia were shaken off and ready to use. Bone marrow derived cell were isolated as described before (Weischenfeldt and Porse 2008). Briefly, bone marrow was flushed from femurs and tibias of C57BL/6 mice and cultured for 1 week in DMEM with supplements (as described above) and L929 fibroblasts conditioned medium. After 7 days in culture adherent cells were approximately 95% pure macrophages and cells were used for experiments. All cells mentioned above were incubated at 37 °C in 5% CO<sub>2</sub>.

#### 3.2.1.2. Human cell culture

The human glioblastoma cells (Tumor 1, Tumor 2, Tumor 3, Tumor 4, and Tumor 5) were derived from human glioblastoma tumor resections which were obtained from glioblastoma patients without any prior clinical history, according to governmental and internal (Charité) rules and regulations. Cortical brain tissue from epilepsy patients was performed according to the rules laid down by the Ethical Committee (Charité, EA1/142/05), and informed consent was obtained according to the Declaration of Helsinki (Lynoe, Sandlund et al. 1991). Freshly isolated human glioblastoma cells were then cultured in RPMI 1640 supplemented with 20 µg/ml EGF, FGF, 200mM glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, and 10% FBS. All cells were maintained in a 37°C incubator with a 5% CO<sub>2</sub> humidified atmosphere. Cells were ready to use after about two to four weeks of culture.

### 3.2.2. Gene knock down and overexpression

#### 3.2.2.1. GDNF knock down with siRNA and shRNA approach

siRNA GDNF (on target plus SMARTpool, Dharmacon) and control scrambled non-targeted siRNA or the siRNA to GAPDH (control SMARTpool, Dharmacon) were transfected with Dharmafect4 (Dharmacon) according to manufacturer's instructions. After 24 hrs of transfection, GL261 cells were injected into the hollow fiber and an aliquot was used for quality control by RT-PCR and ELISA. GDNF transient transfection of GL261 glioma and fibroblast cells was performed using the lipofectamine LTX following the manufacturers protocol (Invitrogen). Plasmid cDNA encoding rat GDNF was kindly provided by Dr. Carlos F. Ibanez at the Karolinska Institute, Sweden. PCDNA3.1 plasmid DNA was used as negative control.

For stable GDNF knockdown, four sequences (sh1, sh2, sh3, and sh4) of GDNF OmicsLink™ shRNA clones and non-targeting scrambled shRNA (shNT) were transfected into GL261 cells according to manufacturer's instructions. Transfected GL261 cells were selected by treating with 5µg/ml puromycin. After selection, GDNF secretion was measured by ELISA in four individual shGDNF GL261 cell preparations and cell proliferation rate was measured by BrdU labeling. We chose the preparation with the lowest GDNF secretion and unchanged cell proliferation for implantation experiments.

| shRNA targeting GDNF | Sequences           |
|----------------------|---------------------|
| <b>sh1</b>           | ATGCCTGAAGATTATCCTG |
| <b>sh2</b>           | AGAAGGCTAACAAGTGACA |
| <b>sh3</b>           | GCCAGTGTTTATCTGATAC |
| <b>sh4</b>           | GCCTTGAGTCCTATGTTAC |

**Table 3.10 List of shRNA targeted to GDNF**

#### 3.2.2.2. CCL5 knock down with siRNA approach

siRNA CCL5 (on target plus SMARTpool, Dharmacon) and control scrambled non-targeted siRNA or the siRNA to GAPDH (control SMARTpool, Dharmacon) were transfected with Dharmafect4 (Dharmacon) according to manufacturer's instructions. CCL5 transient transfection in GL261 and fibroblast cells was performed using the lipofectamine LTX following the manufacturers protocol (Invitrogen).

#### 3.2.2.3. CCL5 cloning

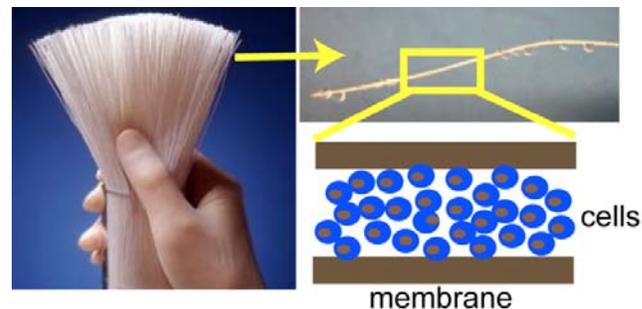
For making the CCL5 overexpression construct, CCL5 ORF was amplified from mouse cDNA using Phusion Polymerase regarding manufacturer guidelines (FINNZYMES OY). The CCL5

ORF was subsequently ligated in EcoRI and XbaI sites in a prior digested mammalian plasmid vector pCDNA3.1 (Invitrogen). The CCL5 construct subsequently identified and transformed into *E. coli* cells, where DNA was harvested using the Qiagen® Hi-Speed® Maxiprep kit®. The successful clones were verified by sequencing (by Stratec company, Berlin) and used for overexpression in the GL261 glioma cell and SCRC fibroblast lines for further use. pCDNA3.1 plasmid DNA was used as negative control for transfection.

### 3.2.3. Hollow fiber cell encapsulation

#### 3.2.3.1. Cell encapsulation

Wild type (wt)-SCRC fibroblast cells, wt-GL261 glioma cells, primary human glioma cells (Tumor 1), GDNF-SCRC fibroblast cells, and siGDNF-GL261 were infused by syringe into a hollow fiber (molecular mass cutoff = 500 kDa) at a density of  $2 \times 10^6$  cells/ml under sterile conditions. Then fibers were sealed at the ends in 5-cm segments. Fibers were cultivated in DMEM or RPMI 1640 with additives for 2 days. One day before measurements or transplantation, the 5-cm long fibers were subsequently cut into length of 5 mm and sealed at both ends.



**Fig 3.1. Illustration of cell encapsulation.** In a density of  $2 \times 10^6$  cells/ml, cell suspension was infused into a hollow fiber by 1 ml syringe.

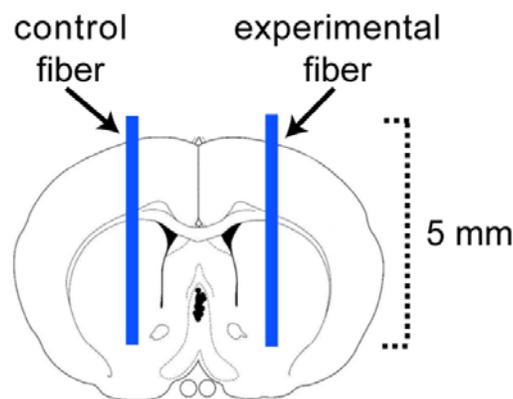
#### 3.2.3.2. Determination of cell proliferation in HF

Cell proliferation was monitored using the Alamar blue assay following manufacturer's recommended procedures. The 5 mm fibers filled with cells were cultured in a 96 well plate. At day 1, 3, 5, and 7 of culture, ten microliter of Alamar blue solution was added to each well to make a 1:10 dilution and incubated for 4 hours at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Wells containing empty fibers were used as control. Absorbance was measured in a spectrometer at 570 and 600 nm. For transplanting the fiber into mouse brain, fibers were also cut into 5 mm of length and the relative cell numbers in the fiber was measured. The fibers with similar cell density were selected for transplantation.

### 3.2.4. Animal model

#### 3.2.4.1. HF inoculation into the mouse brain

Eight- to 10-wk-old wild-type female C57BL/6 were handled according to governmental (LaGeSo) and internal (MDC) rules and regulations (TVV 0268/10). Briefly, mice were anesthetized i.p. with Ketamine and Rompun. The mouse head was placed onto a stereotactic frame and carefully fixed in the flat-skull position. The skin of the skull was disinfected with 10% potassium iodide solution and cut with a scalpel blade. Through a midline incision, a burr hole was made by carefully drilling with a 23-gauge needle tip at 1 mm anterior to the bregma and 1.5 mm both right and left side from the midline. A deep canal was created by inserting a sterile 26-gauge Hamilton syringe with a blunt tip 5 mm ventral from the surface of the dura mater through the left and right burr hole. Then the experimental fiber (e.g. with GL261 cells) was slowly inserted into the right canal and the control fiber (e.g. with SCRC fibroblast cells) was inserted into left canal. The burr holes were closed with bone wax and the skin was sutured with a surgical sewing cone.



**Fig 3.2. Illustration of fiber transplantation.** 5 mm length of control fiber filled with fibroblast and experimental fiber filled with GL261 cells were inserted into left and right hemispheres, respectively.

#### 3.2.4.2. Tumor inoculation into the mouse brain

The tumor inoculation surgical procedure is as described above. Instead of fiber transplantation, 1  $\mu$ l of cell suspension were inoculated at 1.2 mm anterior to the bregma and 2 mm both right and left side from the midline. A group of mice were intracerebral inoculate bilateral either with shNT-GL261 (20000 cells in 1  $\mu$ l, left hemisphere) or with shGDNF-GL261 (20000 cells in 1  $\mu$ l, right hemisphere) GL261 cells. After 14 days, in 10 mice the tumor size was measured by MRI. Survival studies were performed as in one prior study (Glass, Synowitz et al. 2005). Two groups of wild-type C57BL/6 mice received either shNT or shGDNF G261 cells. Survival rate was calculated by Kaplan-Meier method.

### 3.2.5. Tissue preparation, immunofluorescent staining, and image process

#### 3.2.5.1. Tissue preparation

Mouse brains were prepared as previously described (Markovic, Vinnakota et al. 2009). Briefly, mice were perfused with 0.9% normal saline to wash out the blood followed by 4% PFA for fixation. After dehydrated in 30% sucrose, mice brains were embedded into OCT compound and coronal brain sections were cut at 10  $\mu$ m intervals on a cryostat and mounted. Human glioblastoma biopsy specimens were obtained from glioblastoma patients without any prior clinical history, according to governmental and internal (Charité) rules and regulations. Cortical brain tissue from epilepsy patients was performed according to the rules laid down by the Ethical Committee (Charité, EA1/142/05), and informed consent was obtained according to the Declaration of Helsinki (Lynoe, Sandlund et al. 1991).

#### 3.2.5.2. Immunofluorescence staining

Before immunostaining, the mouse brain sections were washed three times with PBS-T (0.1% Triton X-100 and 1X PBS) for permeabilisation and subsequently blocked by incubating with 3% BSA for 1 hour at room temperature. The primary antibodies were applied on sections and incubate for overnight at 4°C. Omission of the primary antibody served as a negative control. Subsequently, secondary antibodies were incubated with sections for 2 hours at room temperature. All antibodies were diluted in blocking buffer (0.1% Triton X-100 and 3 % BSA). Finally, the nuclei were counterstained with DAPI. Glass slides were covered with coverslips and stored at 4°C until used for microscopic analysis.

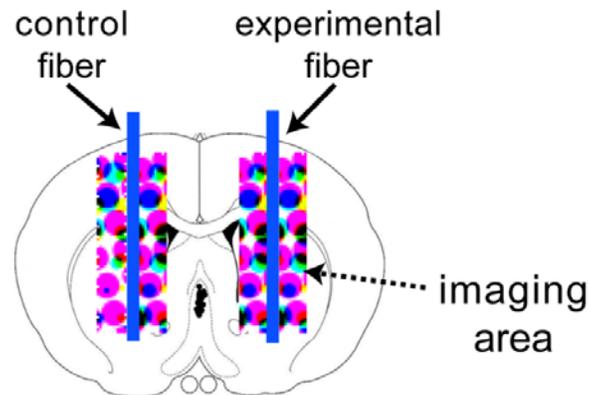
The following primary antibodies were used in different dilutions:

| Antibody                         | Dilutions |
|----------------------------------|-----------|
| <b>Primary antibody</b>          |           |
| Rabbit anti Iba-1                | 1:750     |
| Rabbit anti GFAP                 | 1:1000    |
| Mouse anti vimentin              | 1:100     |
| Rabbit anti GDNF                 | 1:100     |
| <b>Secondary antibody</b>        |           |
| Alexa 488 donkey anti mouse IgG  | 1:200     |
| Alexa 488 donkey anti rabbit IgG | 1:200     |
| Alexa 594 goat anti rabbit IgG   | 1:200     |
| Cy5 donkey anti rabbit IgG       | 1:200     |

**Table 3.11 List of antibody and concentration**

### 3.2.5.3. Confocal imaging and image processing

Confocal microscopy images were taken using a Leica confocal microscopy (TCS SP5, Leica, or LSM710, Zeiss) with 20X, 40X oil, and 63X oil objectives. Images were taken from at least 3 random fields in the tissue surrounding the hollow fiber area of each mouse. Iba-1 positive labeled cells (microglia/macrophages) were counted using Image J software. The intensity of GFAP fluorescent staining (astrocytes) was also measured by Image J without adjusting digital gain.



**Fig 3.3. Illustration of imaging analysis.** Confocal imaging area was selected adjacent the fiber membrane.

### 3.2.6. mRNA and protein expression

#### 3.2.6.1. cDNA and PCR

Total RNA was isolated using an InviTrap® Spin Universal RNA Mini Kit and first-strand cDNA was synthesized with SuperScript II reverse transcriptase by applying 1 µg RNA and oligo-dT primer. PCR was performed with a Takara PCR kit. Sequences for primer sets were as follow:

| Gene                           | Sequence   | Product size |
|--------------------------------|--|--------------|
| <b>gdnf</b>                    | forward 5'-TATGGGATGTCGTGGCTGT-3'<br>reverse 5'-CGTCATCAAACCTGGTCAGGA-3'       | 182          |
| <b>GFR<math>\alpha</math>1</b> | forward 5'-TTCCCACACACGTTTTACCAC-3'<br>reverse 5'- TTTGTGGTTATGTGGCTGGAG-3'    | 144          |
| <b>GFR<math>\alpha</math>2</b> | forward 5'- AAGGCCAACAACTCCAAAGAG-3'<br>reverse 5'- CCAAGGTCACCATCAGGAGT-3'    | 156          |
| <b>CCL5</b>                    | forward 5'- ACCACTCCCTGCTGCTTTG-3'<br>reverse 5'- ACACTTGGCGGTTCTTCG-3'        | 129          |
| <b>CCR1</b>                    | forward 5'- AAGCCGTGCGTCTGATATTTG-3'<br>reverse 5'- TGCTCACACTGATTGGTGAATAG-3' | 118          |

|                |  |     |
|----------------|--|-----|
| <b>CCR3</b>    | forward 5'- ACCCCGTACAACCTGGTTCT-3'<br>reverse 5'- ACCAACAAAGGCGTAGATTACTG-3'    | 159 |
| <b>CCR5</b>    | forward 5'- CAAGACAATCCTGATCGTGCAAG-3'<br>reverse 5'- TCCTACTCCCAAGCTGCATAGAA-3' | 127 |
| <b>b-actin</b> | forward 5'-CCCTGAAGTACCCATTGAA-3'<br>reverse 5'-GTGGACAGTGAGGCCAAGAT'-3'         | 238 |

**Table 3.12 List of primer sequences**

### 3.2.6.2. Western blotting

Cultured cells in 12 well plates or 6 well plates were washed two times with ice-cold PBS before the sample buffer was applied (10 $\mu$ l/cm<sup>2</sup>). Cells were scraped with a rubber policeman and the cell lysates were collected in Eppendorf tubes. Samples were then centrifuged at 13,000 rpm for 10 min and the supernatant was collected; the protein concentration was determined using the BCA protein assay kit. 5X LB sample buffer was mixed with protein lysate and incubated at 95°C for 5 min. Final concentration 20  $\mu$ g of protein were loaded onto SDS-PAGE and electrophoresis was performed at 90 V for 15 min and at 100 V until the lowest molecular marker reached the bottom of the gel. Then gels were removed from the glass plates and equilibrated in transfer buffer for 5 min. Meanwhile, a PVDF membrane was activated by incubation in pure methanol for 1 min and afterwards equilibrated in transfer buffer for another 5 min. The transfer sandwich was covered with the upper electrode and blotted at 100 V for 75 min. The membrane was then washed once in PBS-Tween20 for 5 min and then blocked with 5% BSA blocking buffer for 2 hours. The GDNF antibody was then added in blocking buffer for an overnight incubation at 4°C on a shaker. On the next day, the membrane was washed three times in PBS-Tween20 and incubated with the HRP conjugated anti rabbit antibody for one hour at room temperature. After three washing steps, the ECL reagent was applied for 5 min and the membrane was scanned.

### 3.2.6.3. ELISA

Cells were seeded in 6 well plates and the supernatant was collected after 2 days of culture. GDNF and CCL5 secretion in cell supernatant was determined using specific GDNF or CCL5 ELISA kits according to the manufacturer's protocols (Peprotech). Results are presented as picograms of GDNF or CCL5 per milliliter. To measure whether GDNF is released from HF, 5 mm of fiber with encapsulated cells were placed in a 96 well plate and covered with 200  $\mu$ l of culture medium. Supernatants were collected after 2 days.

### 3.2.7. Cell proliferation assay

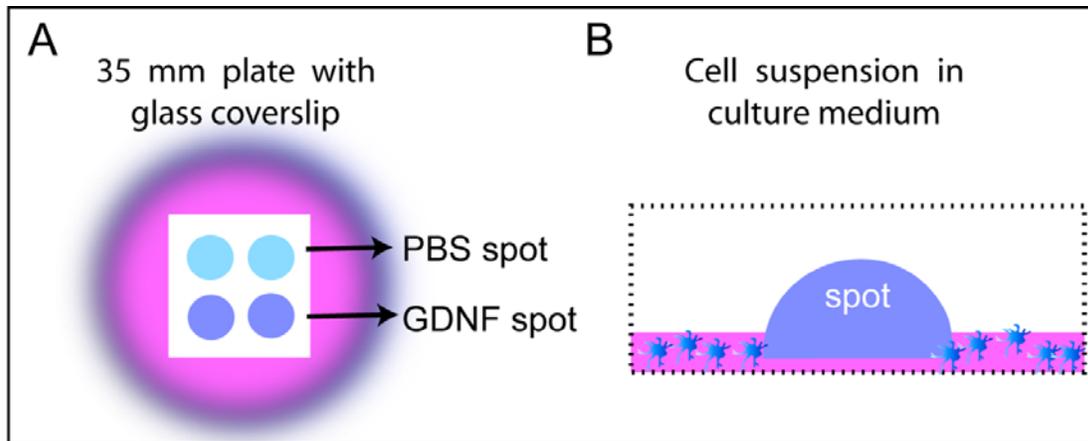
Microglia was suspended at density of 50000 to 200000 cells/ml in DMEM medium with supplements. GL261 cells were suspended at density of 50000 to 100000 cells/ml in serum free DMEM medium. Total 100  $\mu$ l of cell suspension were seeded in 96 well plates. After 15 hrs incubation, 50 nM (for microglia) or 100nM (for GL261) GDNF were added to cells. For microglia proliferation, L929 conditioned medium was added and for GL261 cells, 10% FBS was used as a positive control. After 24hrs, BrdU cell proliferation ELISA (manufacture) was applied according to the manufacturer's protocol. For testing the GDNF knock down effect, sh1-sh4 GDNF treated GL261 glioma cell were plated into 96 well plate one day before the assay.

### 3.2.8. Chemotaxis assay

#### 3.2.8.1. Agarose spot assay

The chemotaxis effect of GDNF on microglia was determined using either the agarose spot assay (Wiggins and Rappoport 2010), Boyden chamber assay, or transwell assay. For the agarose spot assay, 0.1g of low melting point agarose was dissolved in 20ml of PBS to obtain a 0.5% agarose solution. Four spots (each spot 10 $\mu$ l) per dish were placed onto 35-mm glass dishes with two containing GDNF and two containing PBS only (Fig 3.4\_A). One million microglia or GL261 cell suspensions were added to the dishes and incubated at 37°C in 5% CO<sub>2</sub>. After 3 hrs and 7 hrs, microglia was counted under the spot. For GL261 cells, cell migration was determined after 7 hrs and 24 hrs.

For time lapse microscopy, 1 hr after cell seeding and attachment on the cover slip, 25 different positions along the edge of the spots were imaged every 3 minutes over 16 hrs period on a BioStation IM incubation chamber that maintains cells at constant temperature (37 °C) and 5%/95% CO<sub>2</sub>/O<sub>2</sub> for extended periods of time. The images were obtained from different spots using a 20X objective. Data was analyzed using the imaging tools provided by Image J (Tracking, and Chemotaxis and Migration Tool plugins).



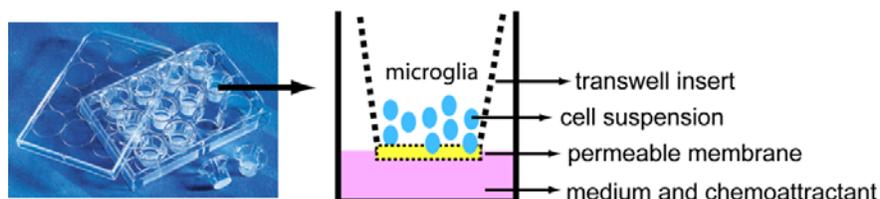
**Fig 3.4. Illustration of agarose spot assay.** **A**, 35-mm Petri dish containing a coverslip in the middle. Two control agarose spot mixed with PBS and the other two mixed with chemoattractant were loaded onto coverslip. **B**, 2 ml of cell suspension was then loaded in Petri dish.

### 3.2.8.2. Boyden chamber assay

For the Boyden chamber assay, a microchemotaxis chamber was used described here in detail (Nolte, Moller et al. 1996). Briefly, microglia (about  $5 \times 10^4$  cells in  $50 \mu\text{l}$ ) were placed into the upper chamber, the lower well was filled with serum free DMEM with or without GDNF. After 6 hrs of incubation, membranes were fixed, cells from the upper surface of membranes were removed with gentle swabbing and the cells on the lower surface of the membranes were stained with Haematoxylin and Eosin. Microglia migration was determined by counting the number of stained cells in at least 5 randomly selected fields on each membrane.

### 3.2.8.3. Transwell assay

The chemotaxis of microglia by GDNF released from glioma cells was determined using a transwell assay (modified Boyden chamber assay). The upper chamber of a transwell insert (pore size  $8 \mu\text{m}$ ) was filled with  $2 \times 10^5$  microglia in suspension with  $500 \mu\text{l}$  of medium (DMEM with 10% FCS and glutamine) and GL261 conditioned medium was placed in a lower chamber of a 24-well culture plate. The transwell plates were incubated for 24 h at  $37^\circ\text{C}$ . Microglial migration was quantified by counting the number of cells that migrated through the membrane using an inverted bright-field microscope (five fields/each well).



**Fig 3.5. Illustration of transwell assay.** 500 microliter cell culture medium with or without chemoattractant were loaded in lower chamber in a 24 well plate. Transwell insert were then placed into each well. Then microglial cell suspension was loaded into insert.

### 3.2.9. F-actin cytoskeleton staining

The F-actin cytoskeleton was visualized using rhodamine phalloidin. Microglia and GL261 glioma cells were cultured on glass coverslips and stimulated with 200ng/ml GDNF or 100ng/ml CCL5. After 6 hrs (for microglia) or 24 hrs (for GL261) of incubation, microglia were fixed with 4% PFA for 15 minutes and after washing steps permeabilized for 5 minutes with PBS-T. The coverslips were washed twice with PBS containing 0.1% Tween-20 and exposed to rhodamine phalloidin (100 nM) in PBS-T for 2 hrs. Cells were counterstained with DAPI (1:1000) for 5 minutes. After wash steps the coverslips were mounted onto glass slides with mounting media.

### 3.2.10. *In vivo* assessment of glioma tumor size by MRI

Tumor size was quantified by MRI. Briefly, anesthesia of the mice was induced with 2.5% isoflurane in an oxygen/air mixture (2:1) with a flow rate of 750 ml/min and maintained at 1.5% to 2% for the rest of the experiments. Respiration rate and body temperature were continuously monitored by monitoring and gating system (Model 1025, Small Animal Instruments). Using a heated circulating water system, the body temperature was maintained at 37 °C throughout the experiments. MR imaging was performed on a 9.4 Tesla small animal MRI system (Biospec 94/20, Bruker) equipped with a cryogenically cooled (to 30 K) radio-frequency probe, designed for imaging of the mouse brain (CryoProbe, 400 MHz). The CryoProbe is a half-cylindrical-shaped, 2-channel, transmit/receive quadrature-driven surface coil, for full mouse-brain coverage with a maximum field of view of approx. 30x20x20mm. T<sub>2</sub>-weighted images (RARE, effective echo time (TE) = 60 ms; repetition time (TR) = 3268 ms; RARE factor = 12) and T<sub>1</sub>-weighted images (MDEFT, echo time (TE) = 3.9 ms, repetition time (TR) = 2500 ms, inversion time = 900 ms) were acquired with the same slice geometry (field of view = 18 x 18 mm, matrix size = 350 x 350, slice thickness = 270 μm, in-plane spatial resolution = 51 μm, 21 coronal slices covering a brain region of 5.67 mm starting at the frontal end of the cerebral cortex (approx. Bregma 3.56 to -2.11 mm (Franklin 2007). Tumor volumes were calculated by manual segmentation using the software *mipav* (<http://mipav.cit.nih.gov>). A region of interest (ROI) following the tumor borders was drawn on the T<sub>2</sub>-weighted images (tumor shows hyperintense). The whole tumor volume was calculated in *mipav* by adding up the voxel volumes within the ROIs of all image slices.

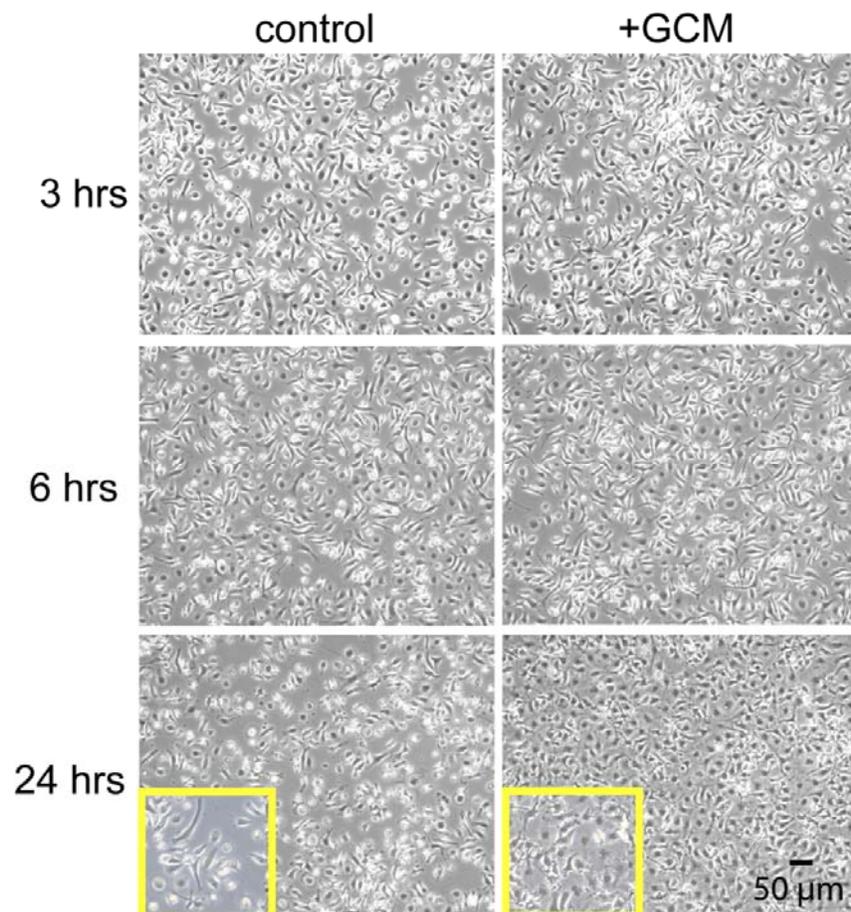
### **3.2.11. Statistical analysis**

All data represent the average of at least triplicate samples. Error bars represent standard error of the mean. Data were analyzed by Student's t test in SPSS and the differences were considered statistically significant at \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

## 4. Results

### 4.1. Glioma secreted soluble factor stimulate microglia

To determine if soluble factors released by glioma cells have impact on microglia (e.g. activation), mouse primary microglia was exposed to glioma-conditioned medium (GCM). Microglial activation can be assessed by morphology changes. Primary microglia in culture exhibit long processes and rod-shaped, and upon activation, they transform into an enlargement of the cell body and acquiring an amoeboid cell shape (Sliwa, Markovic et al. 2007). When microglia culture with normal medium (DMEM+10% FBS) over 24 hrs, they show elongated cell bodies and thin processes. The occurrence of small compact soma bearing long and thin ramified processes normally leads to the ramified, resting microglial phenotype (Fig 4.1, left panels). On the contrary, after 6 hrs cultured with GL261 conditioned medium (DMEM+10% FBS, +GCM), microglia morphology slightly changed and over 24 hrs, the morphology completely switch to activated morphology which appeared several slender branched processes emerged from the soma (Fig 4.1, right panels).

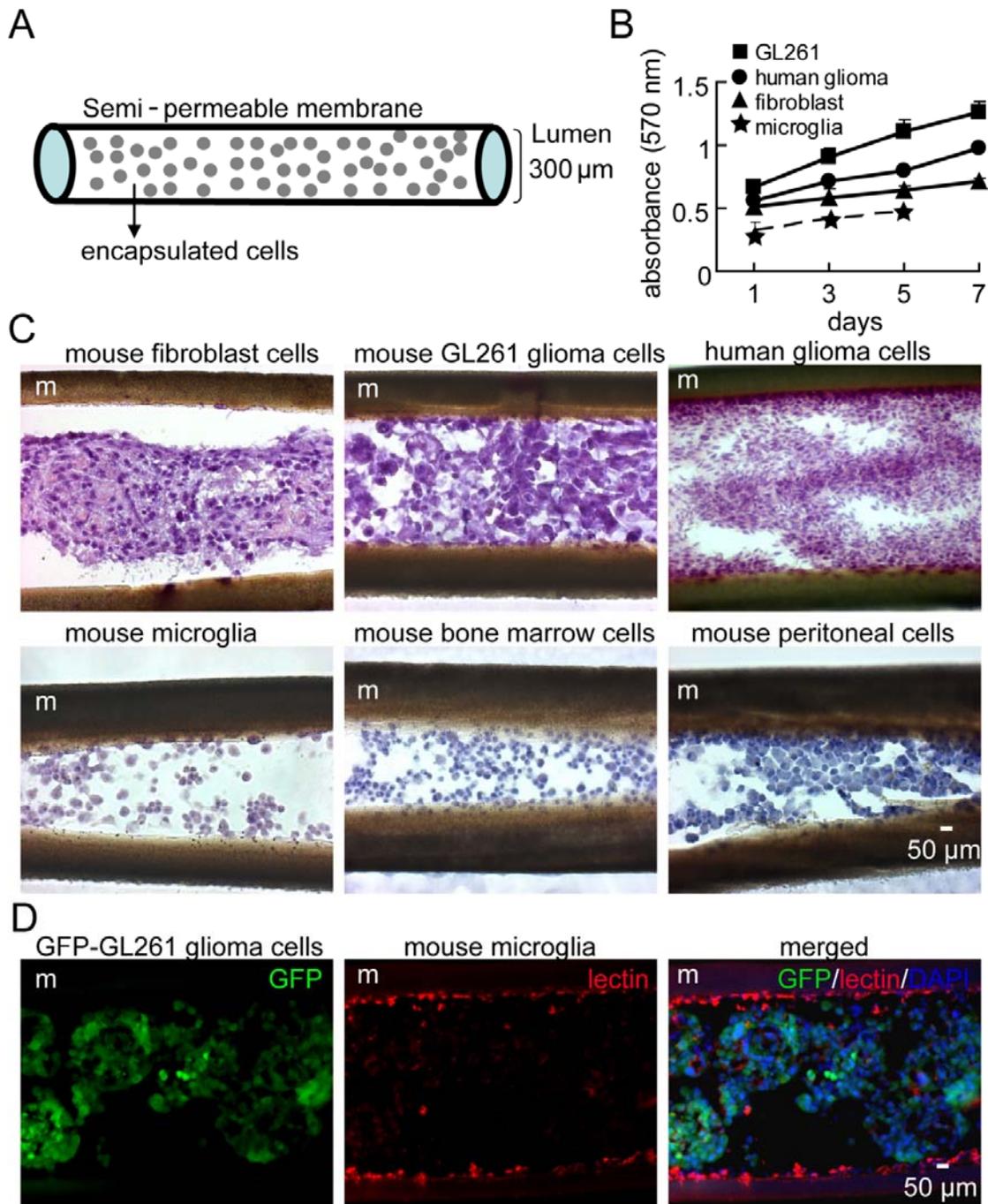


**Fig 4.1. Phase contrast images of mouse microglia.** 1 million mouse primary microglia was cultured in regular medium (as control) or GL261 glioma-conditioned medium (+GCM) in six well plates. After 3, 6, and 24 hrs, images were taken by light microscope. The small yellow box insert depicts higher power images.

## **4.2. Characterization of hollow fiber cell encapsulation model**

### **4.2.1. Cells survived and proliferated in hollow fibers (HFs)**

To explore if soluble factors released from glioma cells have an impact on intrinsic brain cells, I encapsulated glioma cells in a semi permeable HF (Fig 4.2\_A). First, to make sure if cells can survive in HF, I tested different types of cells (GL261 glioma cells, mouse primary microglia, SCRC fibroblast cells, and human glioma cells) for encapsulation. Cell density for encapsulation (ranging from  $1 \times 10^5$  to  $4 \times 10^6$  cells/ml) was also tested in the beginning and observed whether cells survive and even proliferate in HFs. The optimized cell density was  $2 \times 10^6$  cells/ml for all tested cell preparations. Cell proliferation was assayed using the absorbance of Alamar blue at 570 nm as a live cell marker 1, 3, 5, and 7 d after encapsulation. With increasing days after encapsulation, HFs with GL261 glioma cells, human glioma cells, or SCRC fibroblast cells showed an increase in absorbance indicative of an increase in cell number (Fig 4.2\_B). For more diversity, I further encapsulated mouse bone marrow derived macrophage and peritoneal macrophages in HF. Those HFs were further section in cryostat with 10 micro meter thickness. H&E staining of fiber sections demonstrated that the SCRC fibroblast cells, GL261 glioma cells, human glioma cells, mouse microglia, in addition mouse bone marrow derived macrophage and peritoneal macrophages were viable and grew in the HFs after 7 days in culture (Fig 4.2\_C). I also mixed GL261 (with GFP expression) and mouse microglia in 1:10 density for encapsulation to determine how they interact in the HF. After 7 days of encapsulation, most of the microglia was close to the inner membrane and some of them contribute to the glioma cell spherical structure (Fig 4.2\_D).



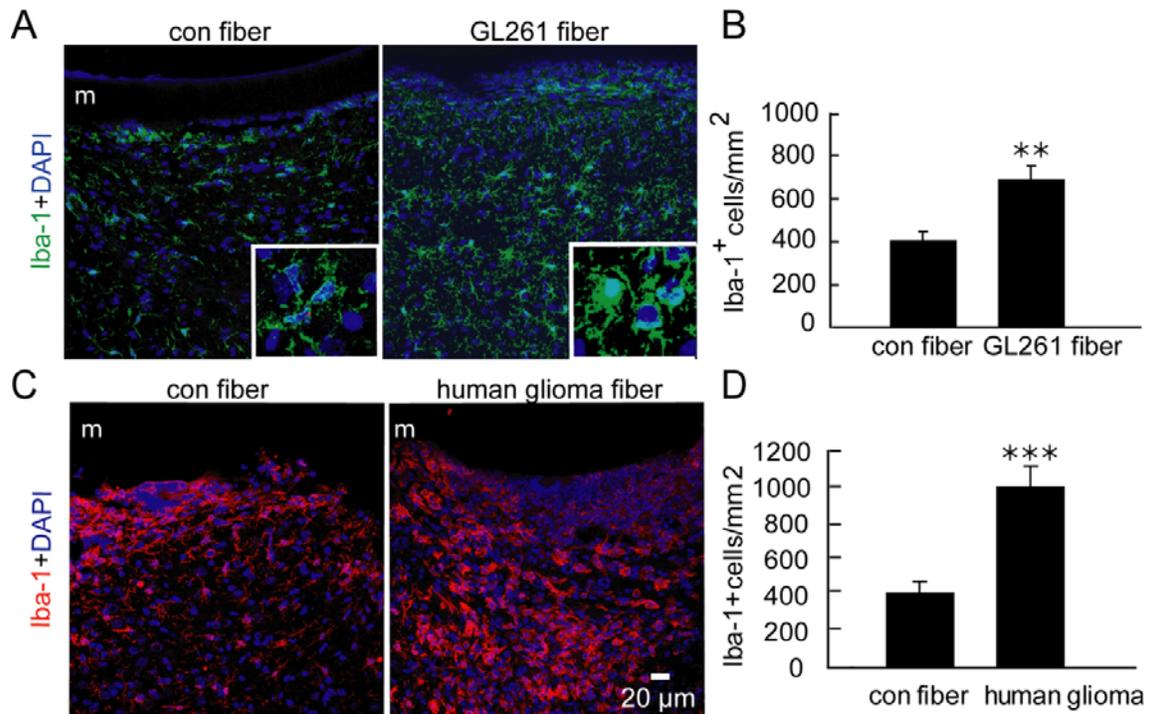
**Fig 4.2. Characterization of hollow fiber cell encapsulation.** **A**, Schematic representation of cell encapsulation in a HF in a cross sectional view. **B**, Cell proliferation in HF on 1, 3, 5, and 7 days, was determined by the Alamar blue assay. Bars show mean $\pm$ s.e.m. from 3 experiments. **C**, Histological longitudinal cross section of HF sections containing encapsulated SCRC mouse embryonic fibroblast cells, mouse GL261 glioma cells, human glioma cells, mouse microglia, bone marrow macrophage, and peritoneal macrophage. Fiber sections were visualized by Hematoxylin and Eosin staining on the 7<sup>th</sup> day of culture. **D**, Longitudinal cross section of HF sections containing encapsulated mixed GFP-GL261 and mouse microglia. Alexa 594 conjugated lectin stain for microglia.

### **4.3. Encapsulated glioma cells induce microgliosis and astrogliosis**

#### **4.3.1. Encapsulated glioma cells attract microglial cells and trigger an amoeboid morphology**

To address the question whether soluble factors released from encapsulated glioma cells induce changes in microglia (e.g. microgliosis), HFs filled with GL261 glioma cells or non-tumorigenic SCRC fibroblast cells were implanted into mouse brain. After 2 weeks histological analysis of the mouse brain sections showed that in HFs containing GL261 glioma, the outer membrane was completely surrounded by Iba-1<sup>+</sup> microglia/macrophages and attracted a higher density than HF-controls (Fig 4.3\_A). The density of microglia surrounding the GL261-filled HF was 1.7 fold higher ( $p < 0.01$ ) as compared to the fibroblast-filled HF (Fig 4.3\_B). There was also a morphological transition of microglia towards an amoeboid phenotype which was more pronounced in cells closer to the HF both in GL261- and fibroblast-filled HF (Fig 4.3\_A, insert).

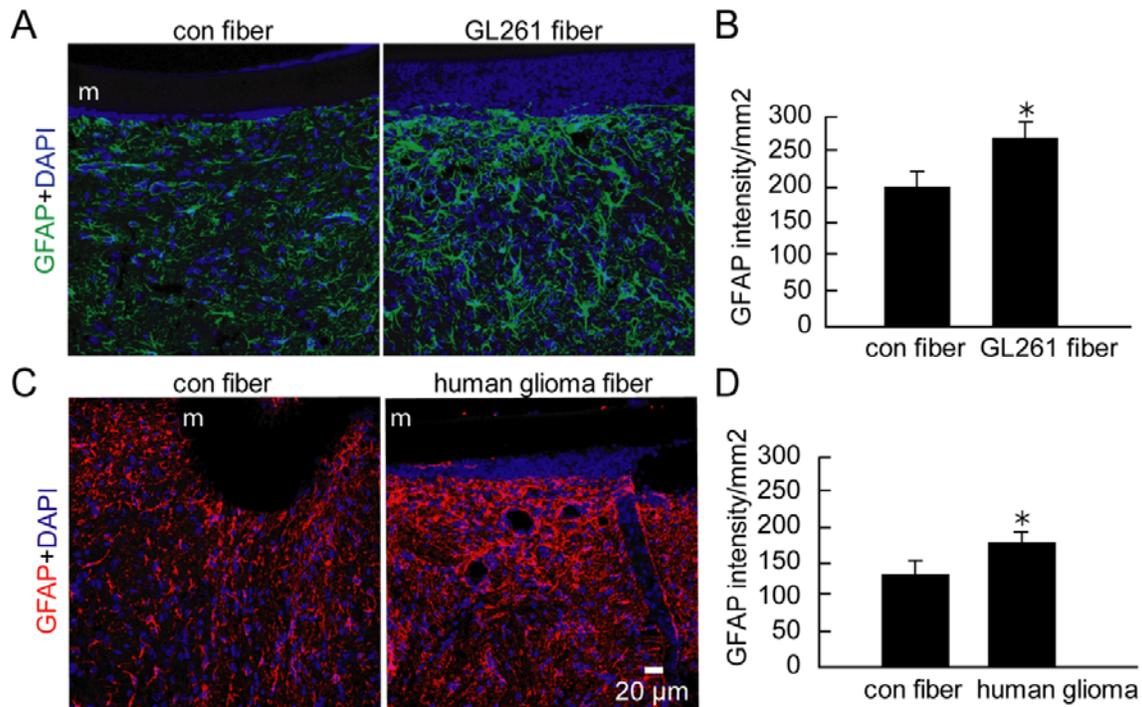
One of the advantages of HF model is that it can prevent the immune response from the host if cells are from different species. Thus, I also encapsulated human glioma cells in HF and implanted them into mouse brain. Similar to the encapsulated mouse glioma cells, Iba-1<sup>+</sup> microglia/macrophages were found to accumulate around the fiber after 2 weeks implantation, indicating microgliosis (Fig 4.3\_C). The density of microglia surrounding the human glioma cells -filled HF was 2.5 fold higher ( $p < 0.001$ ) as compared to the fibroblast-filled HF (Fig 4.3\_D).



**Fig 4.3. Human and mouse glioma cells encapsulated in hollow fibers induce microglia attraction.** **A**, SCRC mouse embryonic fibroblast cells and GL261 glioma cells were filled in HFs and transplanted into the left or right hemisphere, respectively, of a mouse. After 14 days, the mouse was sacrificed and brain sections were stained by immunofluorescence with Iba-1 (green). Cell nuclei were counterstained with DAPI (blue), m indicates fiber membrane. **B**, Quantitative analysis of Iba-1+ cells surrounding fibers (mean±s.e.m. n = 9; \*\*p<0.01) compared with control fiber. **C**, Mouse brain transplanted with fiber encapsulated with human glioma cells into right hemisphere 14 d before the animals were sacrificed and brain sections were stained with Iba-1 (red). Cell nuclei were counterstained with DAPI (blue). **D**: Iba-1+ cells around the HFs were counted. (mean±s.e.m. n = 13; \*\*\*p<0.001) compared with control fiber.

#### 4.3.2. Encapsulated glioma cells trigger astrogliosis

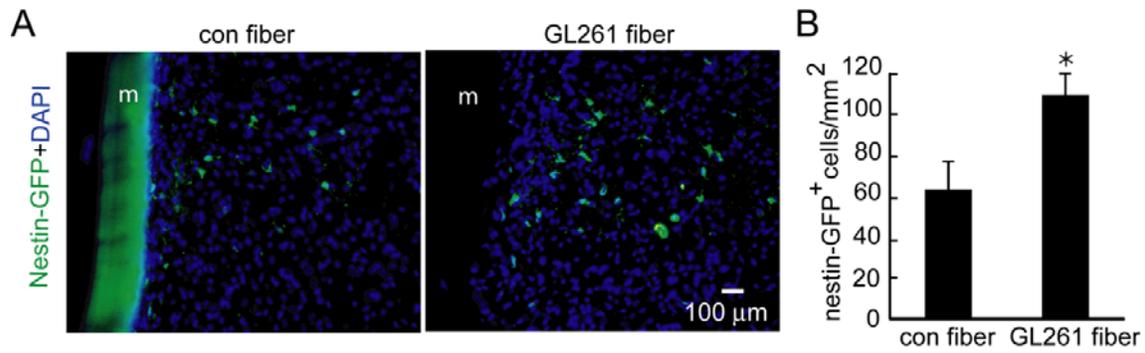
To investigate whether soluble factors from glioma cells also induce astrogliosis, I further measured GFAP immunofluorescence intensity near HF. The density of GFAP-positive astrocytes surrounding GL261 fibers was 1.3 fold ( $p < 0.05$ ) higher when compared with control fibers (Fig 4.4\_B). The morphology of GFAP labeled astrocytes close to the GL261 glioma cell fibers was characterized by a hypertrophic phenotype (increase in the thickness and enlarged soma) that is typical of reactive astrocytes. Astrocytes in the vicinity of control fibers had a normal morphological appearance (Fig 4.4\_A, left). HF encapsulated with human glioma cells resulted in a similar increase in GFAP fluorescence intensity and change in astrocyte morphology (Fig 4.4\_C and D). These findings suggest that glioma cells also influence astrocytes, although not as potently as microglia, via soluble factors.



**Fig 4.4. Human and mouse glioma cells encapsulated in HF induce astrogliosis.** **A**, SCRC mouse embryonic fibroblast cells and GL261 glioma cells were filled in HFs and transplanted into the left or right hemisphere, respectively, of a mouse. After 14 days, the mouse was sacrificed and brain sections were stained by immunofluorescence with GFAP (green). Cell nuclei were counterstained with DAPI (blue), m indicates fiber membrane. **B**, GFAP fluorescence intensity measured from 3 random areas surrounding the fibers (mean±s.e.m. n = 8; \*p<0.05 compared with control). **C**, Mouse brain transplanted with fiber encapsulated with human glioma cells into right hemisphere 14 d before the animals were sacrificed and brain sections were stained with GFAP (red). Cell nuclei were counterstained with DAPI (blue). **D**, GFAP fluorescence intensity measured from randomly selected area surrounding the fibers (mean±s.e.m. n = 13; \*p<0.05 compared with control fiber).

#### 4.4. Encapsulated glioma cells attract NPC

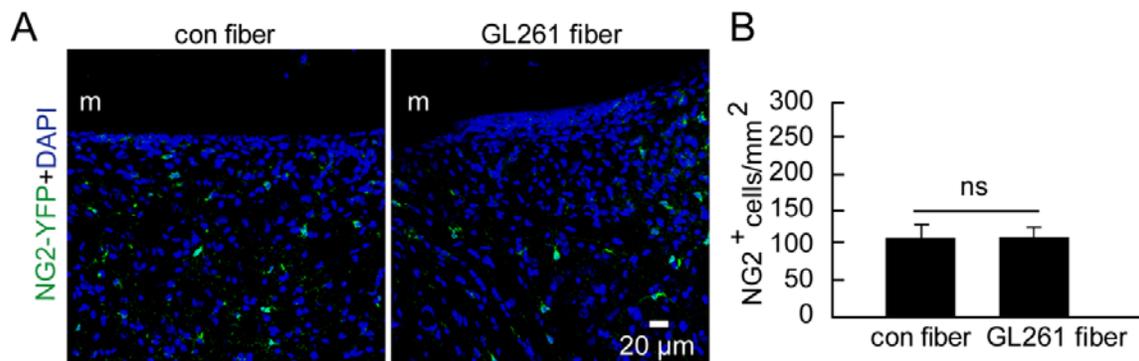
In a murine experimental glioblastoma model, endogenous neural precursors (NPC) have extensive tropism to gliomas. They migrate from the subventricular zone (SVZ) toward the tumor and surround it. To identify whether isolated glioma cells by HF also attract endogenous NPC, I used transgenic mice expressing enhanced green fluorescent protein (GFP) under the control of the Nestin promoter (Glass, Synowitz et al. 2005). The glioblastoma line G261 were culture in HF and then HFs were inoculated into the striatum of the Nestin-GFP transgenic mice (n=8). Fibroblast cells were encapsulated and inoculated into contralateral striatum as controls. Two weeks after inoculation of hollow fiber into the striatum of adult mice, Nestin-GFP-positive cells were found surrounded fiber (Fig 4.5\_A). The number of Nestin-GFP-positive cells are more significant in the hemisphere compared with the contralateral side (Fig 4.5\_B). The results suggest that the GL261 glioma cells release soluble factors from HF and that NPC attraction occurred *in vivo*.



**Fig 4.5. Mouse glioma cells encapsulated in HF induce NPCs attraction.** **A**, SCRC mouse embryonic fibroblast cells and GL261 glioma cells were filled in HF and transplanted into the left or right hemisphere, respectively, of a Nestin- GFP mouse. After 14 days, the mouse was sacrificed and brain sections were stained by immunofluorescence with GFAP (green). Cell nuclei were counterstained with DAPI (blue), m indicates fiber membrane. **B**, Nestin-GFP positive cells were counted surrounding the fibers (mean $\pm$ s.e.m., n = 8; \*p<0.05 compared with control).

#### 4.5. Encapsulated glioma cells has no effect on NG2 cells

So far I have observed the encapsulated glioma cells in HF influenced various intrinsic brain cells such as microglia, astrocytes and NPCs. I would also like to know whether NG2 cells also affected by glioma cells in HF. The control fiber (SCRC fibroblast) and GL261 glioma cells encapsulated fiber were then put into the mouse brain left hemisphere and to the contralateral site, respectively, in NG2-YFP transgenic mice (Karram, Goebbels et al. 2008). After 2 weeks, NG2<sup>+</sup> cells around HF was counted. There was only few NG2-YFP cells in the adjacent brain tissue around both control and GL261 glioma HF (Fig 4.6\_A). The number of NG2<sup>+</sup> cell number was no significant difference between control fiber and glioma fiber (Fig 4.6\_B).

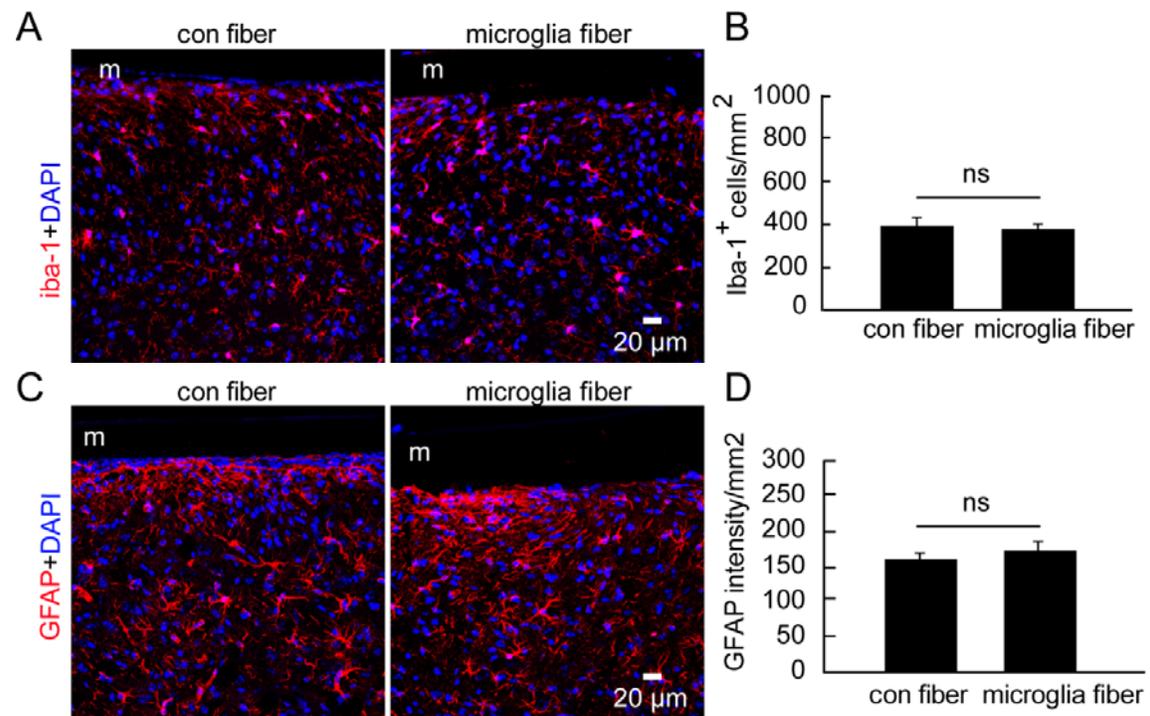


**Fig 4.6. Encapsulated GL261 glioma in fiber does not attract NG2<sup>+</sup> cells.** **A**, Mouse brain transplanted with fiber encapsulated with fibroblast in left hemisphere or with GL261 glioma cells into right hemisphere 14 d before the animals were sacrificed and brain sections were immunofluorescence stained with an antibody to YFP (showed as green). Cell nuclei were

counterstained with DAPI (blue). **B**, NG2-YFP+ cells around the HF were counted. (mean±s.e.m., n=5, ns: not significant)

#### 4.6. Encapsulated microglial cells do not influence the cell attraction

To further approve that the cell attraction was specifically induced by factors which released by glioma cells, I encapsulated microglia into HF and implanted them into mouse brain. Control HF and microglia HF were transplanted into mouse brain left and right hemispheres, respectively. After two weeks of HF inoculation, mice brains were sectioned and stained with Iba-1 and GFAP. The number of attracted microglia was no significant different between control fiber and microglia fiber (Fig 4.7\_A and B). Moreover, GFAP expression intensity close to the HF shows no difference between control and microglia HF (Fig 4.7\_C and D). Collectively, these data suggest that the cell attraction of microglia was specifically induced by glioma cells in HFs.

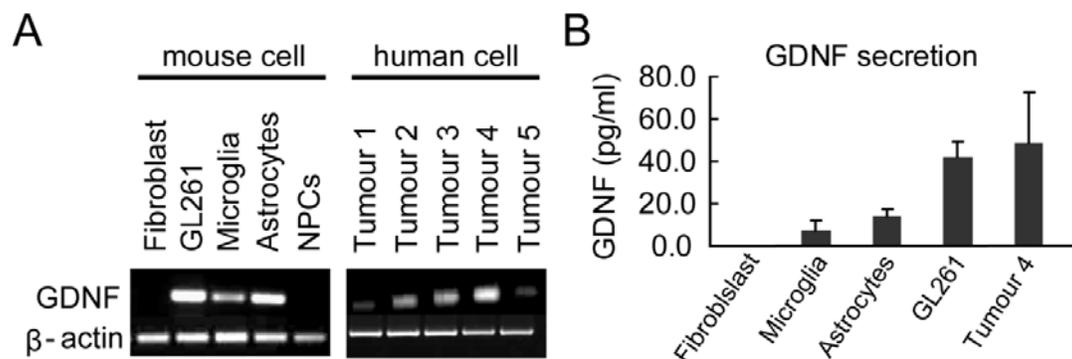


**Fig 4.7. Encapsulated microglia neither attract microglia nor lead to an increase in GFAP expression.** Mouse brain transplanted with enclosed fibroblast in left hemisphere and enclosed microglia into right hemisphere 14 d before the animals were sacrificed. Brain sections were immunofluorescence stained with Iba-1 (A) or GFAP (C). Cell nuclei were counterstained with DAPI (blue). **B**, Iba-1+ cells surround the HFs were counted and the average number per field is shown (mean±s.e.m. from 5 mice). **D**, GFAP fluorescence intensity measured from random area surround fibers (mean mean±s.e.m., n=5, ns: not significant).

## 4.7. GDNF is highly expressed in the mouse and human glioma

### 4.7.1. GDNF is expressed and released in cultured glioma cells

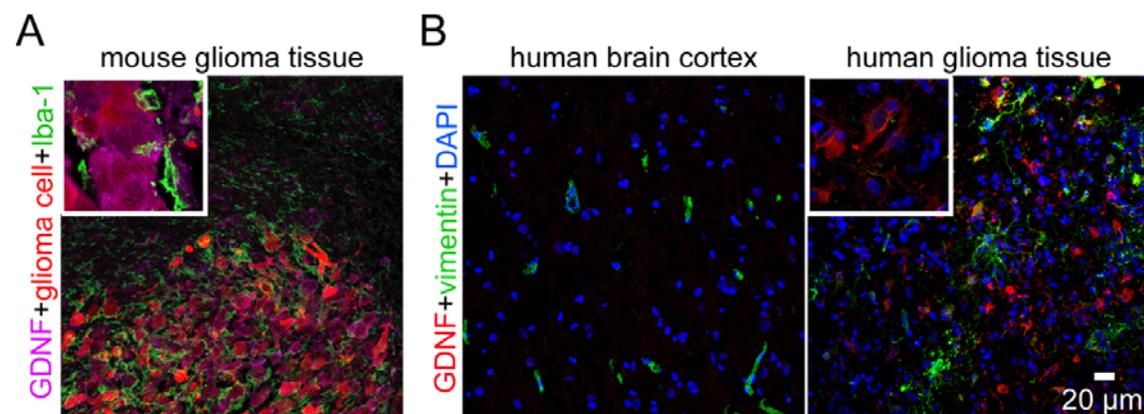
If encapsulated glioma cells attract microglia and induce GFAP expression by releasing soluble factor from HF, which of the soluble factor is involved in this process and how does it work? Earlier study has shown that GDNF is highly expressed in human HG-gliomas (Wiesenhofer, Stockhammer et al. 2000). Thus GDNF could be a potential candidate. To confirm that GDNF is produced and released from the mouse and human glioma cell lines used in my study, GDNF mRNA and protein levels in several mouse and human cells was determined. RT-PCR results showed GL261 cells expressed much higher mRNA levels than mouse astrocytes or microglia while there was no expression in fibroblast and neural precursor cells (Fig 4.8\_A, left). Also in human glioma cells from 5 patients, mRNA for GDNF expression could be verified in all 5 samples (Fig 4.8\_A, right). Since Tumor 4 expressed the highest level of GDNF mRNA, I chose it for the further ELISA measurements. GDNF secretion in the glioma supernatant was determined by ELISA and the results indicated that both human glioma cells (from patient Tumor 4) and GL261 glioma cells secrete GDNF. In microglia and astrocytes, GDNF levels were significantly lower and for SCRC fibroblasts was even below the level of detection (for human glioma Tumor 4,  $48.9 \pm 23.8$  pg/ml; for GL261,  $42.3 \pm 6.8$  pg/ml; for astrocyte,  $13.7 \pm 3.5$  pg/ml; for microglia  $7.6 \pm 4.6$  pg/ml) (Fig 4.8\_B).



**Fig 4.8. GDNF expression in glioma cells. A,** Semi-quantitative RT-PCR of GDNF expression from mouse (left panel) and human cells (right panel): Fibroblast, SCRC mouse embryonic fibroblast; GL261, mouse GL261 glioma cell line; microglia, astrocytes, NPCs, and human glioma cells from 5 individual patients.  $\beta$ -actin was used as a control for mRNA level. **B,** Secreted GDNF protein level was measured by ELISA. Bars represent the mean  $\pm$  s.e.m. from 3 independent experiments.

#### 4.7.2. GDNF is expressed in mouse glioma model and human glioma

After knowing that GDNF is expressed and released from cultured glioma cells, I then asked whether GDNF is expressed in experimental mouse models and human glioma tissues. For induction of glioma in mouse brain, C57BL/6 mice were implanted intracranially with mCherry expressing GL261 cells. After 2 weeks, mouse brain coronary sections were stained with antibodies against GDNF and Iba-1 antibody. As shown in Fig 4.9\_A, high density of Iba-1 positive microglia/macrophage (green) are infiltrated and contribute to tumor mass as described before (Markovic, Vinnakota et al. 2009). Importantly, GDNF is highly co-localized with GL261 cells while I detected no expression in non-tumor area. Human brain tissue from cortex (from an epilepsy surgery) and glioma biopsies were further stained for vimentin and GDNF. Vimentin is known to be expressed in blood vessel in the normal brain tissue as well as in glioma. Indeed, when I stained human brain cortex without tumor, blood vessel showed vimentin expression (Fig 4.9\_B, left). In human glioma tissue, high density of vimentin positive cells was found. Also a high expression of GDNF was detected in glioma (Fig 4.9\_B, right) but not in cortex (Fig 4.9\_B, left). These results show a differential expression of GDNF between glioma cells and the glioma-associated brain cells and suggest that GDNF likely as a candidate molecule mediating glioma-glia cell interaction.

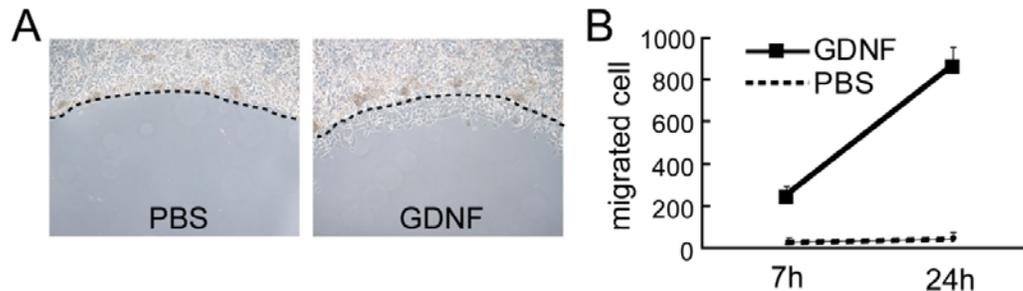


**Fig 4.9. GDNF is expressed in HG glioma.** **A**, Immunofluorescence analysis of tissue sections from a mouse inoculated with mCherry GL261 glioma cells (red) and stained with GDNF (magenta), Iba-1 (green). **B**, Tissue sections from human brain cortex as a control (left panel) and human glioma tissue (right panel) were stained with GDNF (red) and vimentin (green) antibody and DAPI (blue).

#### 4.8. GDNF promote glioma cell migration

Since we now know that GDNF expression is elevated in glioma, I wanted to investigate more closely about what is the role of GDNF in glioma. I first tested if GDNF promotes glioma cell motility. Agarose spots containing either PBS (as control) or 2500 ng/ml GDNF were placed on glass-bottom Petri dishes and one million GL261 cell in suspension were subsequently added. After 7 hrs and 24 hrs incubation time, the agarose spots were

observed under the light microscope (Fig 4.10\_A) and the accumulated GL261 cells to the spots were quantified. GL261 glioma cell invasion under the GDNF-containing agarose spots was significantly higher as compared to the control PBS spots (at 7 hour GDNF spot:  $228 \pm 54$  cells/spot; PBS spot:  $17 \pm 4$  cells/spot,  $p < 0.001$ ) (Fig 4.10\_B).

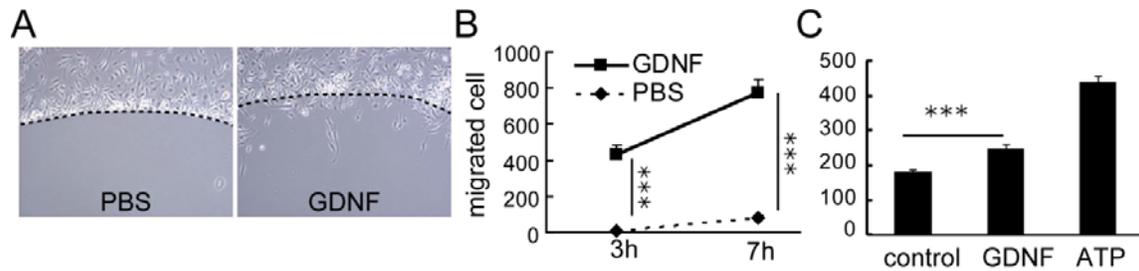


**Fig 4.10. GDNF promote glioma cell chemotaxis.** **A**, GL261 cells was plated on 35 mm plate with bottomed cover slips containing both PBS and GDNF spots. Seven hours after plating, GL261 migrated toward GDNF spots (dashed line depicts border of agarose spot). **B**, Cells migrated under/into the spots were counted. Bars represent the mean  $\pm$  s.e.m. from 3 experiments, \*\*\* $p < 0.001$ .

## 4.9. GDNF is a potent chemoattractant for microglia

### 4.9.1. GDNF induced microglia migration is time dependent

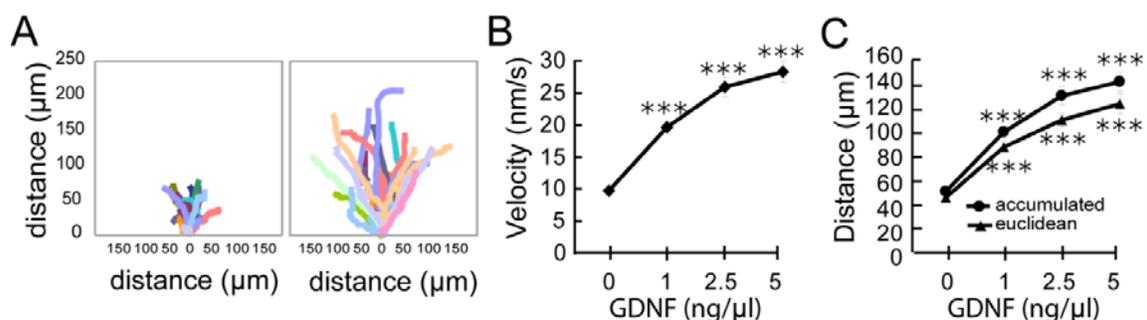
In the view of previous observation that GDNF trigger glioma cell migration, now I aimed to determine the effect of GDNF on microglial chemotactic behavior. I used purified mouse microglial cultures in two different chemotaxis assays, an agarose spot assay and Boyden chamber assay. First, agarose spots containing either PBS (as control) or GDNF were placed on glass-bottomed 35 mm Petri dishes and microglial cells in suspension were subsequently added (Fig 4.11\_A). Compare to glioma cells in culture, microglia cell are more motile. So I chose shorter time point for microglia (3 and 7 hrs). After 3 hrs and 7 hrs incubation time, the agarose spots were observed under the light microscope (Fig 4.11\_A) and the accumulated microglial cells were counted manually. Microglial invasion under the GDNF-containing agarose spots was significantly higher as compared to the control PBS spots (at 3 hour GDNF spot:  $422 \pm 17$  cells/spot; PBS spot:  $8 \pm 1$  cells/spot,  $p < 0.001$ ) (Fig 4.11\_B). From Boyden chamber assay, when applied GDNF (50ng/ml) in the bottom well, higher numbers of microglia penetrated the membrane. ATP is a known strong factor which make microglia more motile so here I used ATP as a positive control (Farber and Kettenmann 2006). At 6 hour, microglia attracted by GDNF:  $247 \pm 14$  cells; control medium:  $181 \pm 8$  cells, ATP:  $440 \pm 16$  cells ( $p < 0.001$ ) (Fig 4.11\_C).



**Fig 4.11. GDNF induces microglia chemotaxis.** **A**, Microglia was plated on 35 mm plate with bottomed cover slips containing both PBS and GDNF spots. Three hours after plating, microglia migrated toward GDNF spots (dashed line depicts border of agarose spot). **B**, Cells migrated under/into the spots were counted. Bars represent the mean $\pm$ s.e.m. from 12 experiments, \*\*\* $p$ <0.001. **C**, Normal culture medium (control), medium contain 50ng/ml GDNF, or 100nM ATP were tested for microglia migration by Boyden chamber assay. Bars represent the mean $\pm$ s.e.m. from 4 experiments, \*\*\* $p$ <0.001.

#### 4.9.2. GDNF induced microglia migration is dose dependent

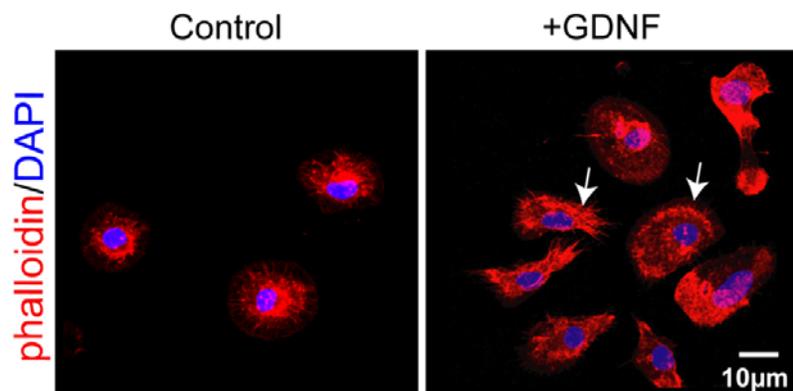
To study the dynamics of microglial invasion, the microglial migration was observed by time-lapse recording over 16 hrs. After time lapse recording, individual cell tracking was manually selected and calculated by Image J software. Microglia migrated faster and over longer distances into the GDNF-containing agarose spots as compared to control (Fig 4.12\_A). Both velocity ( $p$ <0.001, Fig 4.12\_B) and migrated distances ( $p$ <0.001, Fig 4.12\_C) increased in a dose dependent fashion. To study the dynamics of microglial invasion, I analyzed microglial migration by time-lapse recording over 2 hours. Microglia migrated faster and over longer distances into the GDNF-containing agarose spots as compared to control ( $p$ <0.001).



**Fig 4.12. GDNF is involved in microglia chemotaxis.** **A**, Microglia was plated on 35 mm cover slips containing both PBS and GDNF spots. One hour after plating, microglia migration direction was observed by time-lapse imaging and analyzed by Image J software. Each line represents one cell (25 cells for PBS spot and 27 cells for GDNF spot). **B** and **C**, Microglia migration velocity and distance were calculated. Bars represent the mean $\pm$ s.e.m. from 48 cells, \*\*\* $p$ <0.001.

### 4.9.3. GDNF induce microglia chemotaxis is associated with cytoskeleton change

The process of cell migration involves polymerization and depolymerization of the actin filaments during elongation of lamellipodia and filopodia (Lauffenburger and Horwitz 1996). To characterize the migratory response in microglia cultures, process formation and the redistribution of F-actin to the leading edges of processes was observed. After 6 hrs of GDNF treatment (200ng/ml) on microglia, changes in the distribution of cytoskeletal protein (F-actin) in microglia cultures were observed by confocal microscopy. The morphology of untreated microglia showed a minimal number of short processes and actin staining was mainly concentrated in the peri-nuclear area (Fig. 4.13, left). Significant morphological changes were observed after 6 hrs of GDNF treatment. GDNF induced the rearrangement and concentration of F-actin to the end of these processes (Fig 4.13, right, white arrows), suggesting that GDNF induces chemotaxis is related to cytoskeletal changes.

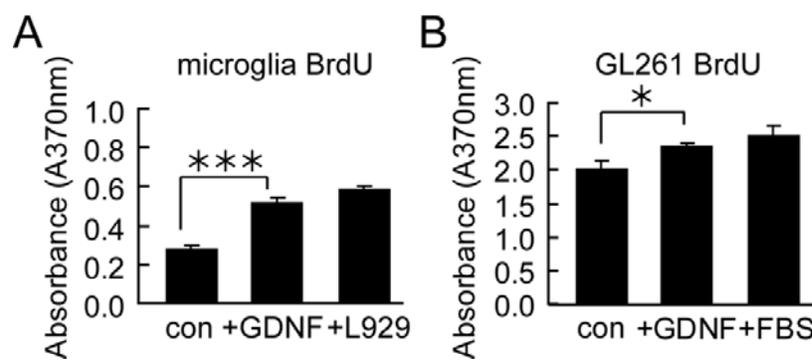


**Fig 4.13. Cytoskeletal changes in mouse microglia in response to GDNF treatment.** Microglia were seeded on glass coverslips and stained for F-actin (phalloidin, red staining) their distribution was analyzed by confocal microscopy. After 6 hrs of GDNF treatment, microglia exhibit focal conglomeration of actin at the leading edge.

### 4.10. GDNF induce cell proliferation

Previous studies reported that GDNF can promote rat C6 glioma cell proliferation (Wiesenhofer, Weis et al. 2000). To test whether GDNF affects the proliferation of microglia and mouse glioma cells, GDNF (50 nM for microglia or 100 nM for GL261) was added into the culture medium, and the proliferation of cells was evaluated by BrdU incorporation. In the beginning I have tested different cell densities in order to optimize the condition (ranging from 50000, 100000, and 200000 cells/ml). Finally I chose 200000 cell/ml. Conditioned medium from L929 fibroblast cell line is known contain macrophage growth factors, therefore, L929

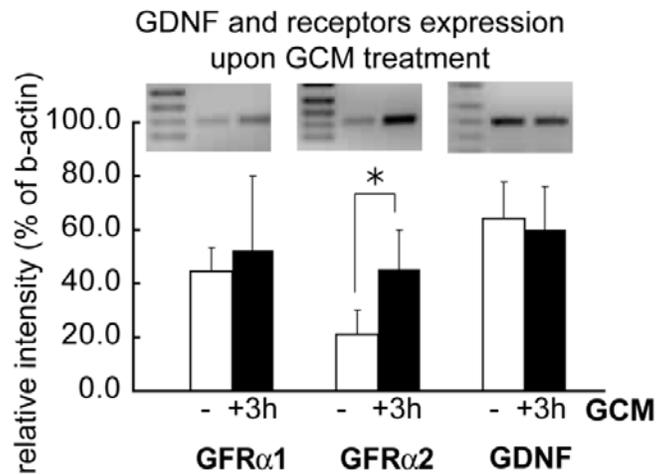
conditioned medium was used here as positive control for microglia proliferation (Sawada, Suzumura et al. 1990). As compared with control, 50nM GDNF promote microglia proliferation by 2 fold higher than control medium (Fig 4.14\_A, \*\*\* $p < 0.001$ ,  $n=3$ ). Because I found that FBS containing medium promote GL261 glioma cell proliferation, they were suspended in serum free medium and the culture medium plus FBS was used for positive control for GL261 proliferation (density: 50000 or 100000 cells/ml). 50000 cells/ml cell density was chosen for the experiment. When compare to control, 100nM GDNF promote GL261 proliferation by 1,1 fold higher than control medium (Fig 4.14\_B, \* $p < 0.05$ ,  $n=3$ ). This result demonstrated that GDNF could significantly stimulate the proliferation of the cultured mouse primary microglia and GL261 glioma cells.



**Fig 4.14. GDNF enhances the proliferation of cultured microglia and glioma cells.** **A**, Quantitative analysis of microglia proliferation as indicated by the percentage of BrdU-positive cells in control and GDNF treated groups. **B**, Quantitative analysis of GL261 cell proliferation as indicated by the percentages of BrdU-positive cells in GDNF treated and control groups. Bars represent the mean $\pm$ s.e.m.,  $n=3$  (\*\*\*)  $p < 0.001$  and \*  $p < 0.05$ ).

#### 4.11. Glioma cell released factor effect on microglia are GDNF receptor mediated

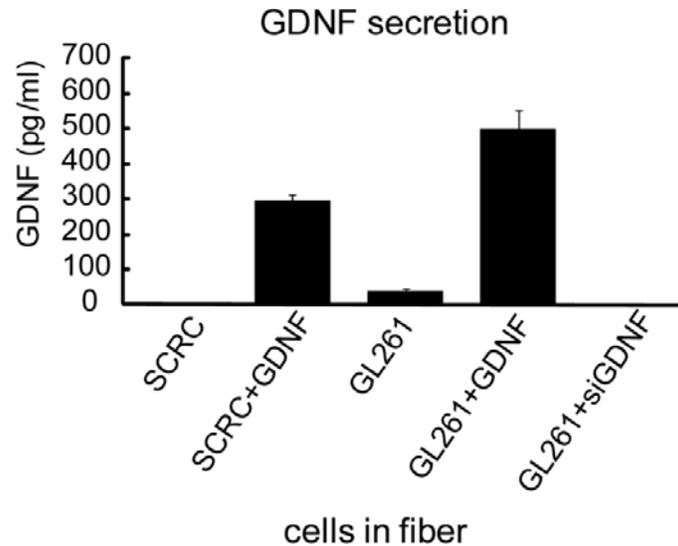
Having established that GDNF promote microglia migration and proliferation, I next asked what is the possible mechanism for the mediation of glioma released factor. The expression of GDNF receptors was examined on microglia after glioma conditioned medium (GCM) treatment. Cultured primary mouse microglia was seeded into 6 well plate in 1 million cell per well density. The next day, medium was changed to control medium or GCM. After 3 hrs of treatment, cell lysate was collected and tested by semi quantitative RT-PCR and tested for GDNF and its receptor expression. GDNF receptor GFR $\alpha$ 1 expression didn't change after 3 hrs of GCM stimulation while GFR $\alpha$ 2 gene expression was upregulated (Fig 4.15, \* $p < 0.05$ ,  $n=5$ ). GDNF gene expression in microglia was no change after 3 hrs of GCM treatment.



**Fig 4.15. GDNF receptors are expressed on microglia upon stimulation by factors released from glioma cells.** RNA was extracted from microglia treated with or without GCM and proceeds with RT-PCR analysis. Bars represent the mean $\pm$ s.e.m. n=5, \* p<0.05).

#### 4.12. GDNF is released from encapsulated glioma cells

As we know from previous results that GDNF is expressed and released from glioma cells (Fig 4.9\_B). It is important to know that in HF model, whether GDNF release can still be detected from encapsulated cells. Thus, I used ELISA to measure the secretion of GDNF from HF. SCRC fibroblast and GL261 cells transfected with GDNF plasmid DNA was used as positive control and GL261 cell transfected with siRNA targeted GDNF was used as negative control. One day after transfection, those cells were encapsulated into HF. After 3 days of culture, cell culture supernatant from HF was collected and examined by GDNF ELISA. As shown in Fig 4.16, no GDNF secretion was detected from wild type SCRC HF while forced GDNF expression in SCRC secreted GDNF. GDNF can be detected from HF entrapped GL261 cells while GDNF knockdown GL261 did not release GDNF from HF.

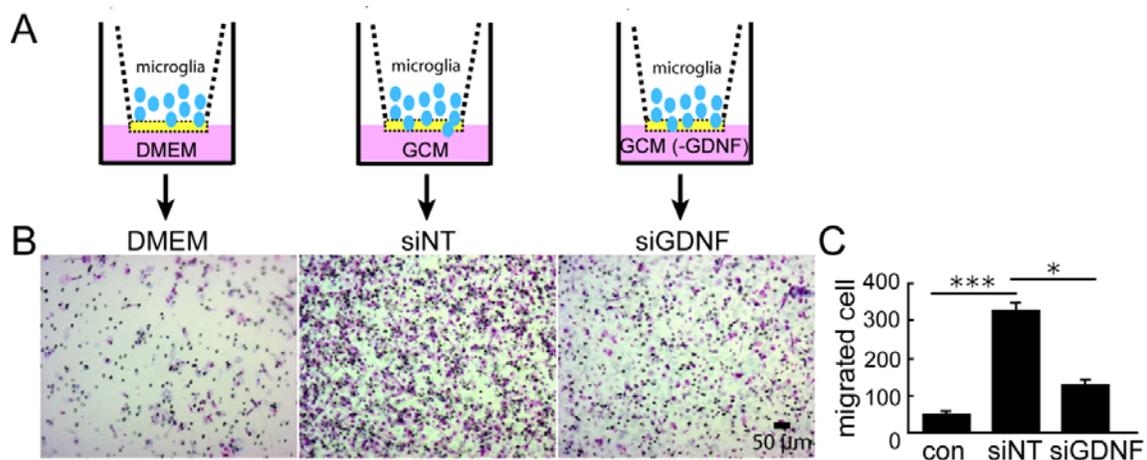


**Fig 4.16. Release of GDNF from encapsulated cells.** 5mm of cell encapsulated HF were incubated with culture medium for 3 days. The supernatant was collected and tested for GDNF release from HF. Bars represent the mean $\pm$ s.e.m. n=3

## 4.13. GDNF plays a key role in regulating microglia attraction

### 4.13.1. GDNF knockdown attenuate microglia migration *in vitro*

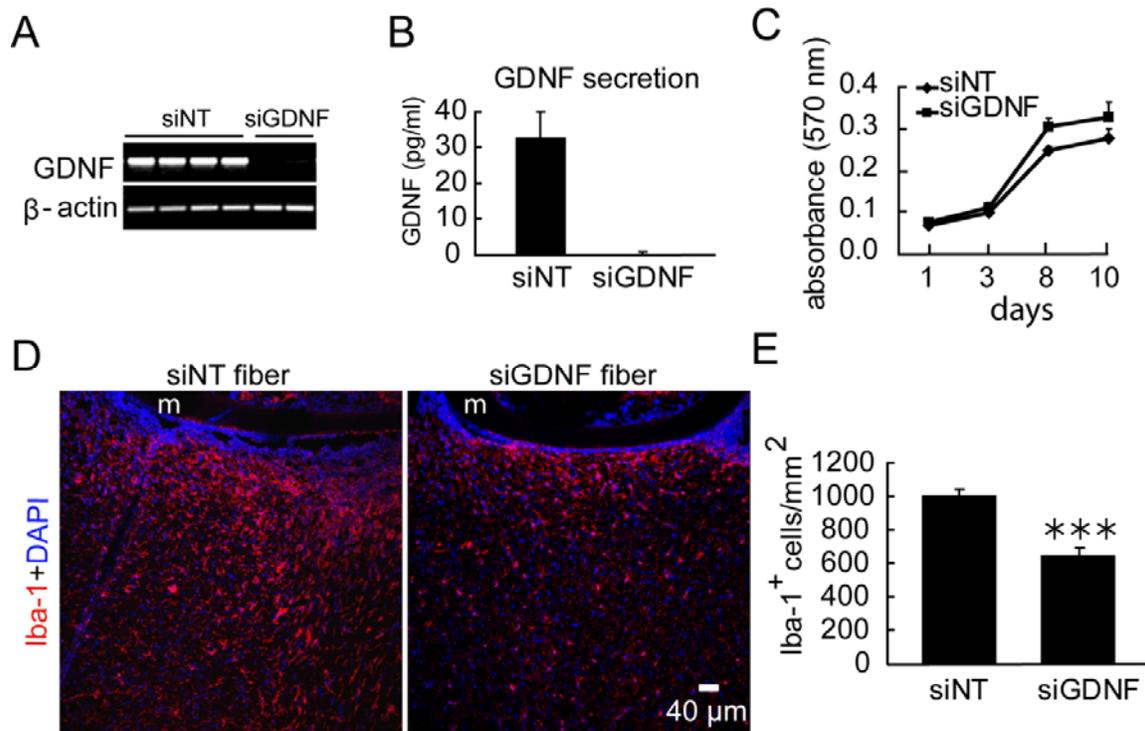
To verify the hypothesis that GDNF secreted from GL261 cells influences microglia migration, the transwell assay was applied. Glioma conditioned medium (GCM) was collected from either non targeted siRNA (siNT, control) or siGDNF (siRNA against GDNF) GL261 cells and applied to the lower transwell chamber (Fig 4.17\_A). After 24 hrs incubation, the transwell membrane was stained with H&E (Fig 4.17\_A and B) and the density of the number of microglia migrating across the membrane was counted. During a 24 hrs transwell migration assay in control DMEM medium, microglia show only basal migration, while GCM from GL261 cells transfected with non-targeting siRNA induced microglia migration (Fig 4.17\_C, \*\*\* $p < 0.001$ , n=3). GCM from GDNF siRNA knockdown GL261 significantly reduced the microglia chemotaxis as compared to medium from siNT transfected glioma cells (Fig 4.17\_C, \* $p < 0.05$ , n=3). Based on these data, I suggest that GDNF released from glioma cells induces microglia chemoattraction.



**Fig 4.17. GDNF knockdown in GL261 reduced microglia chemotaxis.** **A**, Microglia were seeded in a transwell plate in the presence of control medium (DMEM with 10% FBS), glioma conditioned medium from non-targeted siRNA transfected GL261 cells (GCM), or glioma conditioned medium from GDNF-targeted siRNA transfected GL261 cells (GCM -GDNF). The transwell plate was then incubated for 24 h. **B**, Then migrating microglia in the opposite side of the filter were fixed and stained. **C**, Migrated microglia was counted from at least 5 fields. Bars represent the mean  $\pm$  s.e.m. from 3 experiments (\*  $p < 0.05$  and \*\*\* $p < 0.001$ ).

#### 4.13.2. GDNF knockdown attenuate microglia migration *in vivo*

To address whether GDNF mediates glioma-glia cell interactions *in vivo*, I knocked down GDNF expression in glioma cells by siRNA mediated gene knockdown (siGDNF). After two days of siGDNF transfection in GL261 glioma cells, the GDNF expression in GL261 was reduced by >80% (Fig 4.18\_A) and the GDNF secretion was completely diminished, while the control non-targeting siRNA did not affect GDNF expression and secretion (Fig 4.18\_B). GDNF-silenced GL261 glioma cells were then encapsulated into HF. Fig 4.18\_C shows that the knock down of GDNF by siRNA did not affect the glioma cell viability and growth in HF. Next, those HF containing control (siNT) and siGDNF GL261 glioma were cultured *in vitro* and then implanted into mouse brains as described above. Six days after implantation, the mice were sacrificed and the brain sections were stained for Iba-1 (Fig 4.18\_D). GDNF knocked down GL261 in HF reduced microglia attraction ( $643 \pm 47$  cells/mm<sup>2</sup>) by 1.6 fold ( $p < 0.001$ ) when compared to control GL261 cells (siNT,  $998 \pm 48$  cells/mm<sup>2</sup>) (Fig 4.18\_E).

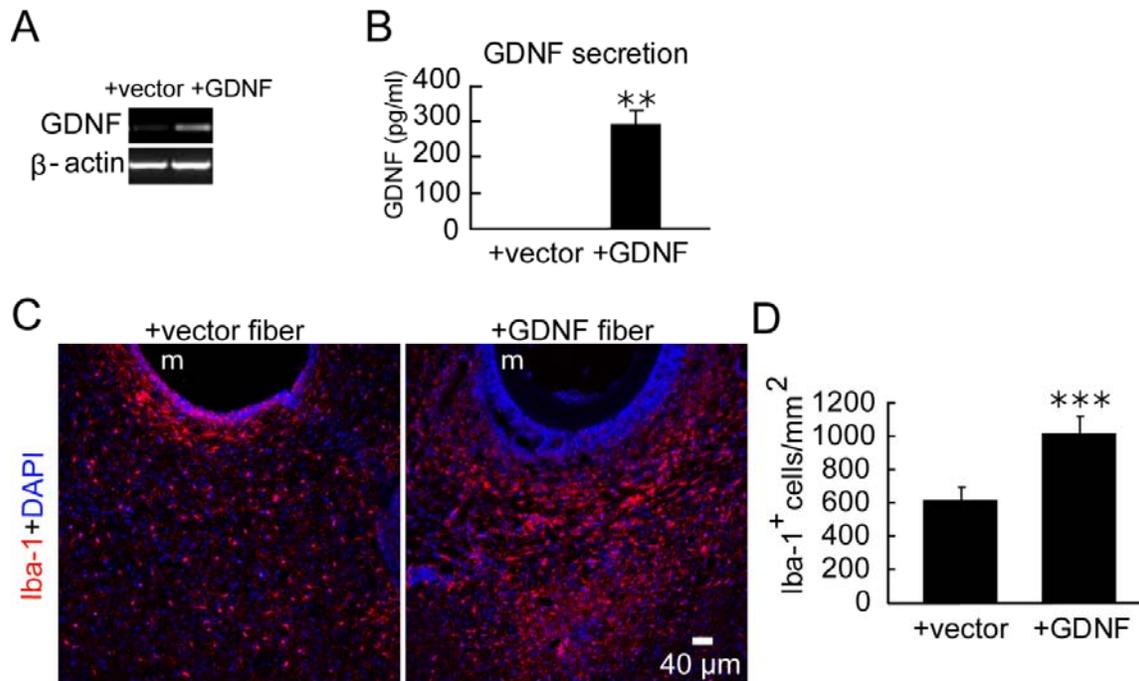


**Fig 4.18. Knockdown of GDNF reduces glioma induced microglia attraction.** **A**, Semi-quantitative RT-PCR of GDNF shows GDNF silencing. GL261 glioma cells were transfected with non targeting (siNT) or GDNF (siGDNF) siRNA. After 3 days, cell lysates were analyzed by RT-PCR.  $\beta$ -actin was used as a control for mRNA level. **B**, GDNF secretion from siRNA treated GL261 cells was also analyzed by ELISA. **C**, Time course of GL261 cell proliferation after siRNA transfection was estimated using an Alamar blue assay. The results represent the mean $\pm$ s.e.m. from 5 fibers. **D**, After transfection with either siNT (left panel) or siGDNF (right panel), enclosed GL261 cells in HF were implanted into the mouse brain (left and right hemisphere, respectively). After 6 days of implantation, mice were sacrificed and brain sections were stained with Iba-1 (red) antibody. Cell nuclei were counterstained with DAPI (blue). m indicates HF membrane. **E**, Attracted microglia surrounding HF were counted (mean $\pm$ s.e.m. n = 11; \*\*\*p<0.001) compared with control fiber.

#### 4.13.3. Forced GDNF over expression in fibroblast induce microglia migration

If GDNF is really a crucial factor that affecting microglia attraction, then GDNF overexpression in non tumor cell should give rise to induction of microglia attraction. Next, I investigated whether over-expression of GDNF by non-glioma cells could attract microglial cells. SCRC fibroblast cells were transfected with either empty vector (+vector) or GDNF vector (+GDNF). Three days after transfection, GDNF mRNA expression in fibroblast cells was increased by >80% (Fig 4.19\_A). GDNF secretion was significantly increased in the culture supernatant of GDNF-transfected cells as compared to cells transfected with control vector ( $296.7\pm 16.8$  pg/ml, p<0.01) (Fig 4.19\_B). The two types of SCRC cells were encapsulated into HF, implanted into mouse brain and analyzed after 6 days. Staining for Iba-1 revealed that the microglial density increased by 1.7 fold (p<0.01) around HF filled with

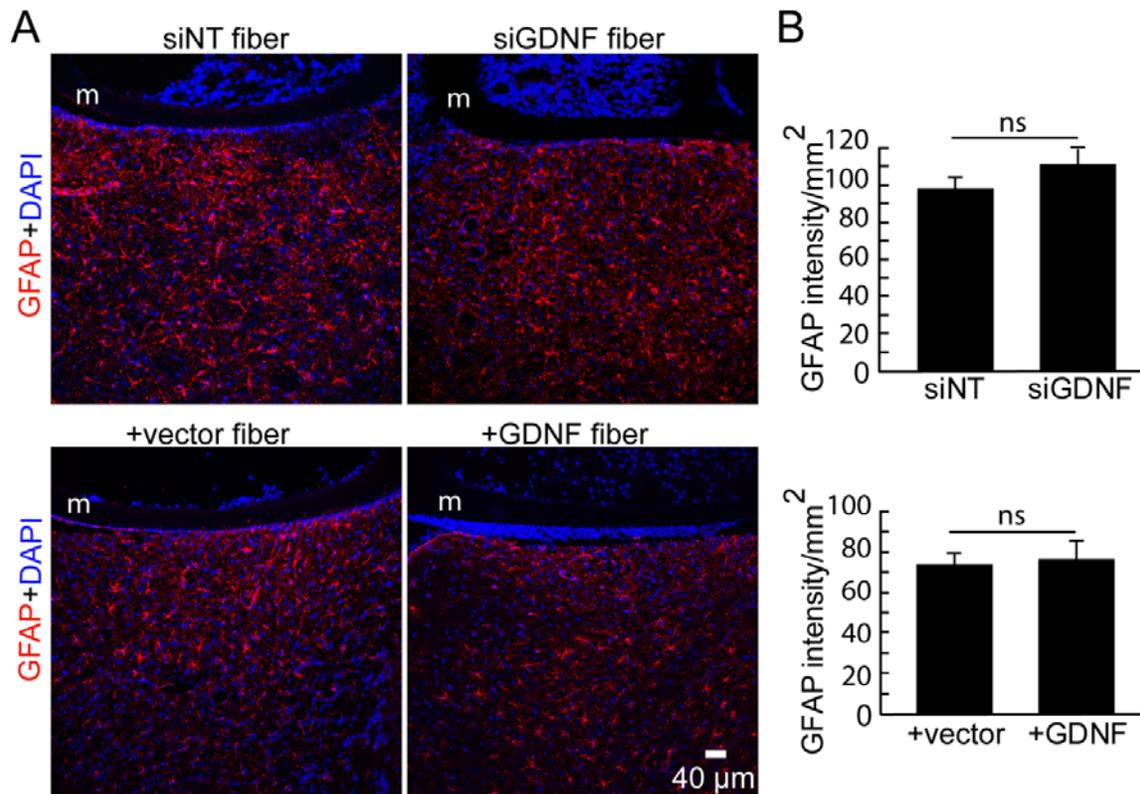
SCRC fibroblast overexpressing GDNF (Fig 4.19\_C), as compared to HF filled with control SCRC fibroblast (Fig 4.19\_D). These results indicate that GDNF released from GL261 glioma cells and GDNF overexpressing non-tumorigenic fibroblasts attract microglial cells.



**Fig 4.19. Forced GDNF expression in fibroblast cells induced microglia attraction.** **A**, SCRC fibroblast cells were transfected with control vector or GDNF. After 3 days, cell lysates were analyzed by RT-PCR. PCR shows the over-expression of GDNF after GDNF transfection. **B**, GDNF secretion was detected by ELISA and shows increased secretion. **C**, After transfection with either control (left panel) or GDNF vector (right panel), enclosed fibroblast cells in HFs were implanted into mouse brain (left and right hemisphere, respectively). After 6 days of transplantation, mice were sacrificed and brain sections were stained with Iba-1 antibody. Cell nuclei were counterstained with DAPI (blue). m indicates HF membrane. **D**, Attracted microglia surrounding fibers were counted (mean $\pm$ s.e.m. n = 5; \*\*\* p<0.001).

#### 4.14. GDNF does not affect glioma induced astrogliosis

Since GDNF has the effect on microgliosis, I next evaluated the impact of GDNF on astrogliosis. I again used two different controls. HF filled with control (siNT) versus GDNF knockdown (siGDNF) GL261 glioma cells as well as SCRC fibroblast control (+vector) versus GDNF overexpressed fibroblast (+GDNF) were implanted into mouse brain (Fig 4.20\_A). There was no significant difference in GFAP intensity and cell morphology in cells surrounding both siNT and siGDNF fibers (Fig 4.20\_B, upper panel) and in cells surrounding SCRC+vector and SCRC+GDNF fiber (Fig 4.20\_B, lower panel). This result indicated that neither down-regulation of GDNF in glioma cells nor over expression of GDNF in non-tumorigenic fibroblast cells influenced the glioma induced astrogliosis.

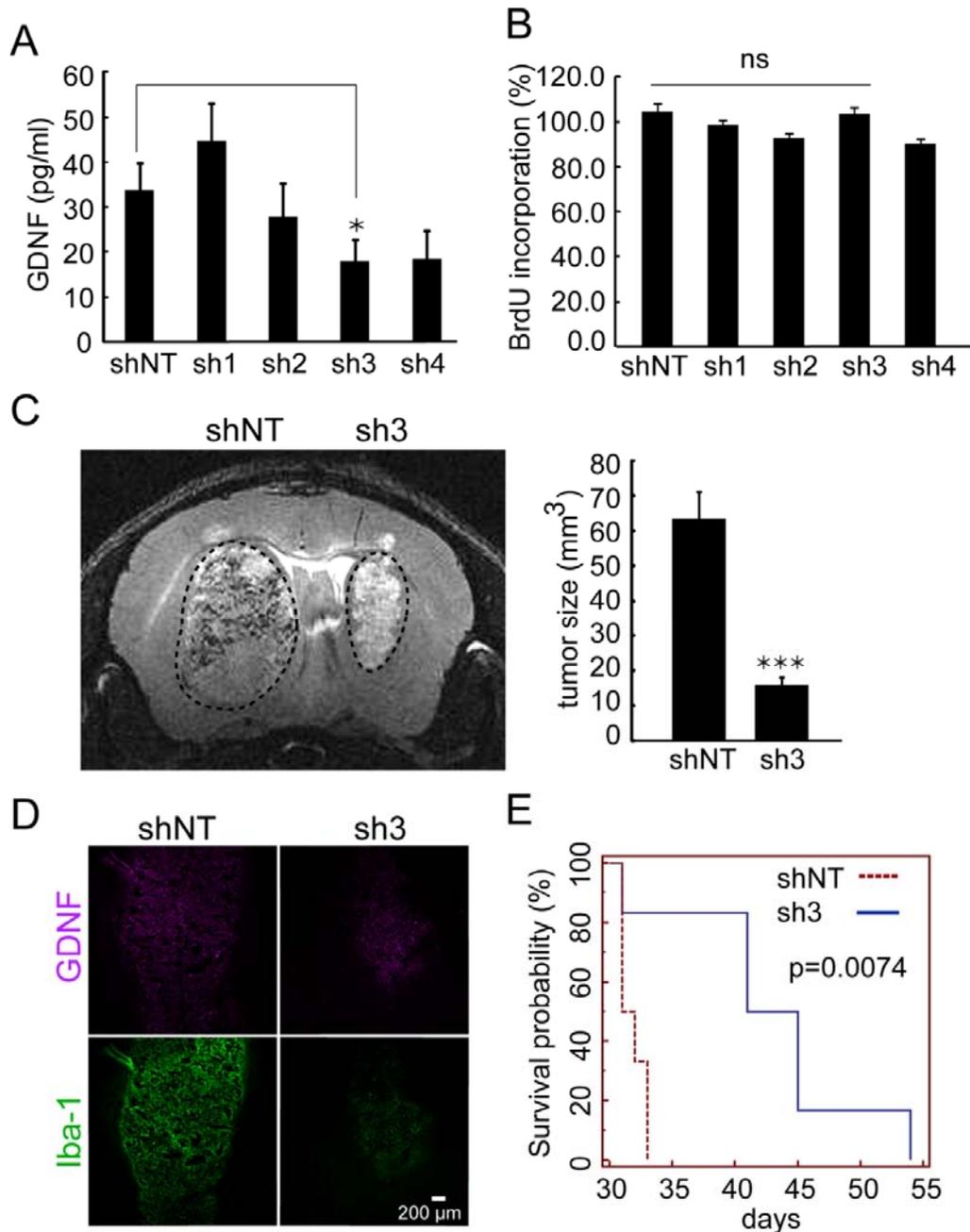


**Fig 4.20. GDNF does not alter the astrogliosis.** **A**, Enclosed GL261 cells in HF<sub>s</sub> transfected with either siNT (left panel) or siGDNF (right panel) were implanted into mouse brain (left and right hemisphere, respectively). Enclosed fibroblast transfected with either control or GDNF vector were implanted into mouse brain (left and right hemisphere, respectively). After 6 days, mice were sacrificed and brain sections were stained for GFAP (red). Cell nuclei were counterstained with DAPI (blue). **B**, The GFAP fluorescence intensity surrounding HF<sub>s</sub> did not differ between the experimental groups.

#### 4.15. Silencing of GDNF in GL261 reduced the tumor size of mice bearing glioma

I then asked what's the impact of GDNF silencing in glioma progression. Since the siRNA transfection was only stable for up to 6 days, and I wanted to investigate later time points of tumor growth and also the survival of the animals after GDNF knock down. To determine whether GDNF is a critical factor for microglia mediated glioma growth, a stably blockade of GDNF production (shRNA) was generated in the glioma cell line. I first studied the influence of stable GDNF knockdown on tumor growth with the help of in vivo magnetic resonance imaging (MRI). I tested four different commercial GDNF shRNA sequences. Data from ELISA revealed that three shRNAs (sh2, sh3 and sh4) decreased GDNF secretion levels after one month of clone selection and the targeting sequence of sh3 reduced GDNF levels most efficient ( $p < 0.05$ ) (Fig 4.21\_A). The GDNF knockdown effect can be observed up to 2 months of selection. To test if permanent GDNF knockdown would affect cell proliferation, BrdU

incorporation of shRNA transfected GL261 cells was tested. Data revealed that sh1, sh2, and sh4 slightly affected GL261 cell proliferation while sh3 did not (Fig 4.21\_B). Thus, I decided to use sh3 for further *in vivo* experiments. Non-targeting shRNA (shNT) and GDNF-targeting shRNA-3 (sh3) transfected GL261 were then injected into mouse brain. After two weeks, tumor size was visualized and measured by MRI (Fig 4.21\_C, left panel). Knockdown of GDNF in GL261 cells significantly reduced glioma tumor size (Fig 4.21\_C, right panel,  $p < 0.001$ ). To address the correlation of GDNF reduction in glioma and less attraction of microglia, mouse brain sections were stained with GDNF and Iba-1. Fig 4.21\_D shows that successful knock down by shRNA is confirmed by reduction of GDNF immunofluorescence and reduced microglia attraction compared to control glioma (inoculated with shNT GL261). Furthermore, the improved survival in the group of mice that were inoculated with GDNF knockdown (sh3) GL261 was observed (Fig 4.21\_E,  $p = 0.0074$ ). Altogether, these *in vivo* data suggest that GDNF is a crucial factor for microglia attraction and glioma progression.

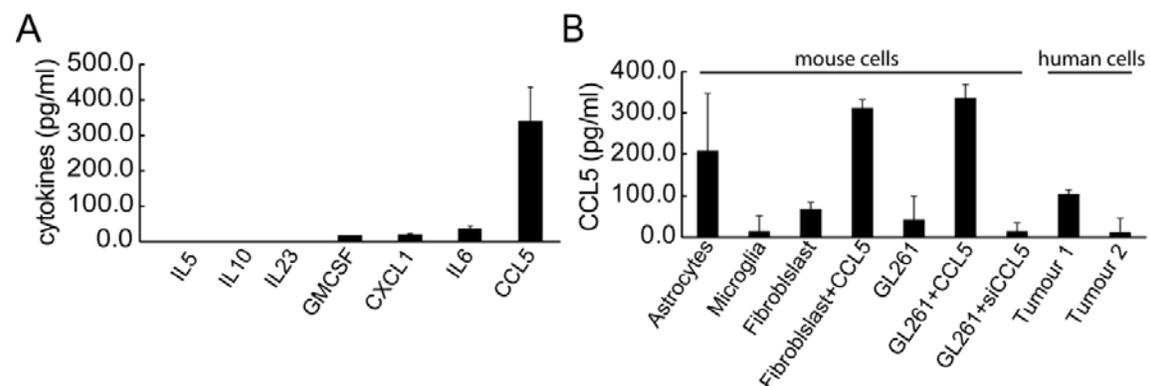


**Fig 4.21. GDNF knockdown decrease glioma tumor size and prolong survival.** **A**, GDNF knockdown in GL261 cells was accomplished by transfecting four different constructs and measuring GDNF secretion after one month of selection (\* $p < 0.05$ ). **B**, 24 hours BrdU incorporation of shNT, sh1, sh2, sh3 and sh4 transfected GL261 cells. Bars represented percentage over control (shNT). Because of significant lower GDNF secretion and no change in proliferation we chose sh3 for further experiments. **C**, Coronal T<sub>2</sub>-weighted *in vivo* MR image of the mouse brain depicting intracranial HG-gliomas (borders highlighted by dashed lines). Note the difference in tumor size between the control GL261 (shNT, left hemisphere) and the GDNF knockdown GL261 (sh3, right hemisphere). Tumor size was calculated by manual segmentation of the tumor borders on the MR images ( $n = 10$ , \*\*\* $p < 0.001$ ). **D**, Immunofluorescence analysis. Mouse brain inoculated with both shNT and shGDNF GL261 in each brain hemisphere was fixed and cryosectioned. 20  $\mu$ m section were stained with GDNF (magenta) and Iba-1 (green). **E**, Kaplan-Meier survival curves of glioma cell implanted mice. Survival of mice was tracked following inoculation of  $2 \times 10^4$  shNT or shGDNF GL261 cells ( $n = 6$  each group,  $p = 0.0074$ ).

## 4.16. Glioma released CCL5 is another potential potent chemoattractant for microglia

### 4.16.1. CCL5 secretion in different cell types including glioma cells

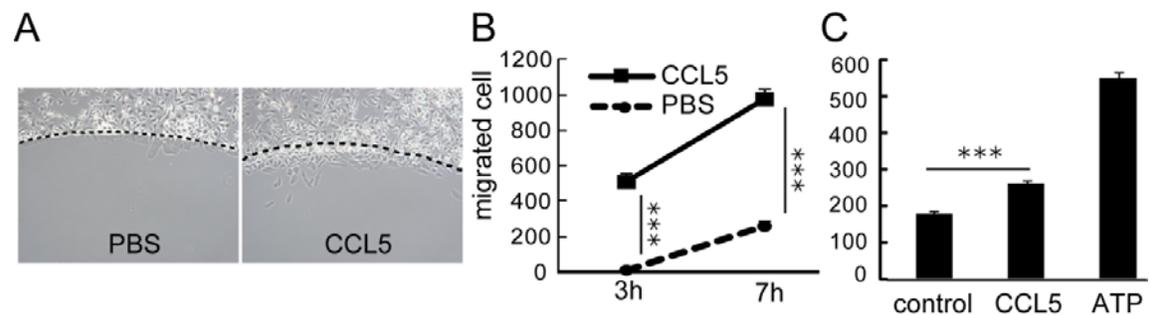
Data from our group and others confirmed that microglia/macrophages are found within human GBM where they likely promote tumor progression (Markovic, Glass et al. 2005; Coniglio, Eugenin et al. 2012). I have found GDNF to be one, but not the only chemoattractant for microglia in the glioma context. To further investigate more about the glioma released factor in the relation of microglia infiltration, rather than GDNF, I studied, whether there are any other soluble factors in this chemotaxis theme. To have a broader view about what kind of factors are released by glioma cells, our cooperater did the analysis by FACS based method (CBA method). Total 8 different GL261 conditioned media samples were determined the cytokine secretion and protein levels. I found that GMCSF, CXCL1, IL6 and CCL5 can be detected (Fig 4.22\_A). From these 7 cytokines, the production of CCL5 from GL261 was the highest. Notably, just earlier this year there was a report showed that CCL5 is highly expressed in mouse and human glioma cell lines (Pham, Luo et al. 2012). I then focused on CCL5 as a potential target in my study. I further used CCL5 ELISA kit to compare the CCL5 production between different cell types. SCRC fibroblast cell and GL261 glioma cells were transfected with CCL5 to overexpress CCL5 as positive control. Mouse microglia secret low level of CCL5 while astrocyte secret 10 fold higher. The level of CCL5 secretion in GL261 lower when CCL5 over-expressed fibroblast cell (Fibroblast+CCL5) and GL261 cell (GL261+CCL5) were used as positive controls. GL261 cells were further transfected with siRNA target to CCL5. The CCL5 secretion was decreased in CCL5 knockdown GL261 (GL261+siCCL5) compare to wt GL261 (GL261) (Fig 4.22\_B). Furthermore, human glioma cells (Tumor1 and 2) also release CCL5 but Tumor 1 secrets a higher amount of CCL5.



**Fig 4.22. CCL5 is secreted in glioma cells.** **A**, Quantification of cytokine and chemokine release by CBA in GL261 conditioned medium. **B**, Cell supernatant from mouse cells and human glioma cells were tested the secretion of CCL5. Bars represent the mean $\pm$ s.e.m. from 3 independent experiments.

#### 4.16.2. CCL5 induces microglia migration

After knowing CCL5 is released by cultured GL261 cells, fibroblast cells, astrocytes, and human glioma cells, but not microglia, I sought to determine the effect of CCL5 on microglial chemotactic behavior. Agarose spot assay was applied. Agarose spots containing either PBS (as control) or CCL5 in 2500 ng/ml were placed on glass-bottomed Petri dishes and microglial cells in suspension were subsequently added (Fig 4.23\_A). After 3 hrs and 7 hrs incubation time, the agarose spots were observed under the microscope and the accumulated microglial cells were quantified. Microglial invade into and migrate under the CCL5-containing agarose spots was significantly higher as compared to the control spots (at 3 hour GDNF spot: 509 $\pm$ 52 cells/spot; PBS spot: 11 $\pm$ 4 cells/spot,  $p < 0.001$ ) (Fig 4.23\_B). Data was confirmed by another chemotaxis assay- Boyden chamber assay. When applied CCL5 (20ng/ml) in the bottom well, higher numbers of microglia penetrated the membrane. At 6 hour, microglia attracted by CCL5: 260 $\pm$ 11 cells; control medium: 179 $\pm$ 7 cells, ATP: 553 $\pm$ 15 cells ( $p < 0.001$ ) (Fig 4.23\_C).

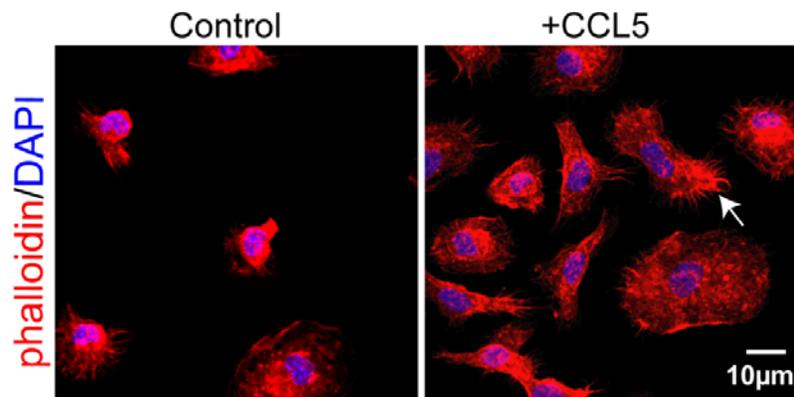


**Fig 4.23. CCL5 induces microglia chemotaxis.** **A**, One million of mouse primary microglia was plated on 35 mm plate with bottomed cover slips containing both PBS and CCL5 spots. Three hours after plating, microglia migrated toward CCL5 spots (dashed line depicts border of agarose spot) or PBS spot. **B**, Microglia migrated under/into the spots were counted at 3 and 7 hrs. Bars represent the mean $\pm$ s.e.m. from 5 experiments, \*\*\* $p < 0.001$ . **C**, Normal culture medium (control), medium contain 20ng/ml CCL5, or 100nM ATP were tested for microglia migration by Boyden chamber assay. Bars represent the mean $\pm$ s.e.m. from 3 experiments, \*\*\* $p < 0.001$ .

#### 4.16.3. CCL5 induces microglia cytoskeleton change

To further characterize the CCL5 induced migratory response in microglia cultures, the morphology and the redistribution of F-actin to the leading edges of processes was observed. Recombinant CCL5 (200ng/ml) was added to microglia culture medium, and after 6 hrs the changes in the distribution of cytoskeletal protein (F-actin) in microglia cultures were

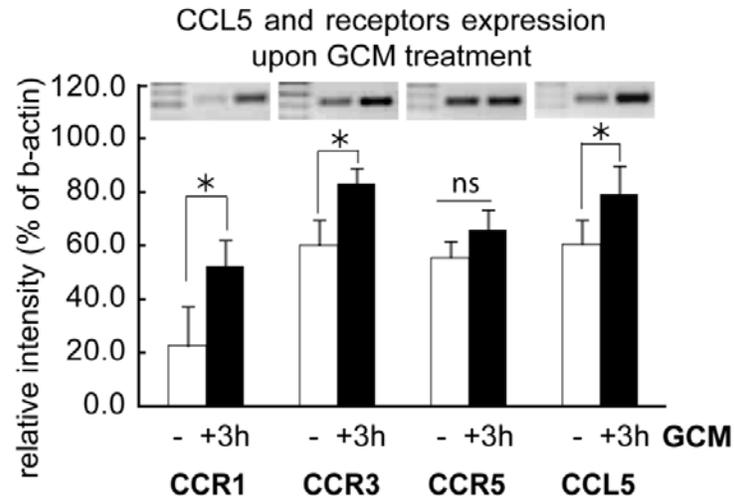
observed by confocal microscopy after phalloidin staining. The morphology of untreated microglia (control) showed a minimal number of short processes and F-actin staining was mainly concentrated in the peri-nuclear area (Fig. 4.24). On the contrary, significant morphological changes were observed after 6 hrs of CCL5 treatment. White arrow indicates that CCL5 induced the rearrangement of F-actin to the end of these processes (Fig 4.24, arrow), suggesting that CCL5 induces chemotaxis is related to cytoskeletal changes.



**Fig 4.24. Cytoskeletal changes in mouse microglia in response to CCL5 treatment.** Microglia were seeded on glass coverslips and stained for f-actin (phalloidin, red staining) their distribution was analyzed by confocal microscopy. After 6 hrs of CCL5 treatment, microglia exhibit focal conglomeration of actin at the leading edge (arrow).

#### 4.16.4. Microglial CCL5 receptors are regulated upon stimulation by factors released from glioma cells

Having established that CCL5 promotes microglia migration, I next asked if CCL5 receptors are involved in the process. There are three known chemokine receptors (CCR1, CCR3, and CCR5) are specific for CCL5. Thus, I first tested the expression of CCR1, CCR3, and CCR5 in microglia with or without glioma conditioned medium (GCM) treatment. Cultured mouse microglia was seeded into 6 well plate. The next day, medium was changed to control medium or GCM. After 3 hrs, cell lysate were collected and test by semi quantitative RT-PCR. There was no change of chemokine receptor CCR5 expression after 3hrs of GCM stimulation while the expression of CCR1 and CCR3 were upregulated (Fig 4.25.).

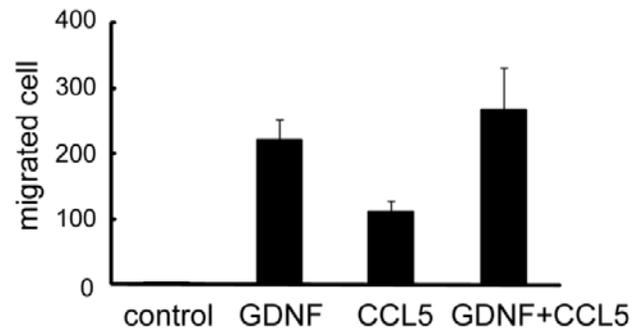


**Fig 4.25. CCL5 and its receptors expression on microglia upon GCM stimulation.** RNA extracted from microglia treated with or without GCM was tested by RT-PCR analysis. Bars represent the mean $\pm$ s.e.m. from 5 experiments, \*  $p < 0.05$ ).

#### 4.16.5. GDNF has stronger chemotaxis effect on microglia

To verify whether GDNF or CCL5 has stronger chemotaxis effect on microglia, agarose spot assay was used. 1 control (+PBS) spot, 1 GDNF (2500 ng/ml) spot, 1 CCL5 (2500ng/ml) spot, and 1 mixed (GDNF1250 ng/ml+CCL5 1250ng/ml) spot were placed in one Petri dish. Microglia suspension was then placed into dish. After 3 hrs, microglia migrated under each spot were counted. Microglial invasion under the GDNF agarose spots was higher as compared to the control spots (at 3 hour GDNF spot:  $223 \pm 30$  cells/spot; PBS spot:  $1 \pm 0.1$  cells/spot) (Fig 4.26). Furthermore, when compare to CCL5, microglia migrated more towards GDNF spot.

Although both GDNF and CCL5 have chemotaxis effect on microglia in the chemotaxis assays, however, this result may imply that GDNF has stronger effect on attracting microglia. ELISA data (Fig 4.22\_B) also revealed that CCL5 is not only secreted by GL261, but also astrocytes and fibroblast cells. Overall, I showed here the *in vitro* data, which might give a hint for future experiments. In my thesis, I wanted to focus on the role of GDNF in microglia infiltration and the implication in glioma, although it is also worthy to study further about the role of CCL5.



**Fig 4.26. GDNF has stronger chemotaxis effect.** One million of mouse primary microglia was plated on 35 mm plate with bottomed cover slips containing control (PBS) GDNF, CCL5, and mixed spots. Three hours after plating, microglia migrated under spots were counted. Bars represent the mean  $\pm$  s.e.m. from 4 experiments.

## 5. Discussion

### 5.1. Hollow fiber as a model for monitoring cell-cell interaction via soluble factor

In tumor microenvironment, it has been known that there are two possible ways of cellular interaction involved: first, through the released soluble factor (acts as a mediator) which essential for cell survival to affect neighboring cells resulting in tumor progression; second, cells are also able to communicate via gap-junctions or receptors, which cell-cell contact is necessary (Zhang, Couldwell et al. 1999). However, how to distinguish these two different ways of communication? In the glioma context, several crucial characters are involved in tumor progression: different types of cells, cell-derived molecule, factors derived from interacting cells. Recently, we and others identified microglial cells and intrinsic neural progenitor cells as an important interaction partners for glioma cells (Chirasani, Sternjak et al. 2010). These observations again raise a number of questions: Which cells in the brain are influenced by glioma cells?; Do glioma induce a change in the adjacent brain tissue that is responsible for tumor progression?; What soluble factor is secreted to attract intrinsic or peripheral cells? Although microglia has been found as important regulatory cells in glioma invasion, the possible key molecules mediating microglia recruitment, trafficking and proliferation remain unclear. Even if a number of glioma released soluble factors such as MCP-1 (CCL-2), CX3CL1, and SDF-1 (CXCL-12) which cause recruitment of microglia toward glioma have been currently described *in vitro* (Kenig, Alonso et al.; Zhao, Najbauer et al. 2008; Magge, Malik et al. 2009), *in vivo* experiments are missing.

Hollow fibers (HF) were widely used for delivering therapeutic substance from cell source *in vivo*. To avoid interfering (cell-cell-contact) of the implanted cells they are separated from the host immune system by enclosure within a semi permeable membrane. Bidirectional diffusion through the membrane of oxygen, nutrients, metabolic products, as well as biological products of encapsulated cells to ensure their survival and functional activities. Specifically speaking of generally HF application for CNS, the cells are transplanted into target CNS tissue by inserting using stereotactic technique. Accumulating evidence suggests that such an approach may be useful in treatments of trauma or functional deficits associated with neurodegenerative disorders such as Parkinson's (Li, Williams et al. 1999; Kim, Hitchcock et al. 2005), Alzheimer's, Huntington's disease, and multiple sclerosis (Emerich and Salzberg 2001) by providing cell-derived therapeutic molecules or by providing trophic factor producing cells such as dopaminergic neurons. Though, originally the strategy of cell encapsulation in hollow fiber was used as tool for gene or cell therapy (Visted, Bjerkvig et al.

2001; Han, Sun et al. 2009). In my study, I adapted and modified cell encapsulation method in order to exclude the cell-cell contact factor and simplify the complex cellular crosstalk in glioma by blocking the cell contact.

Herein, by using HF approach, this study provides a tool for studying the direct interaction of the brain environment on biology of the transplanted cells. Orthotopic tumor induction is suitable for modeling clinical pathology because it mimics the entire growth process of primary tumor, local tumor infiltration, and metastatic translocation. Comparing to that point, an key advantage of HF approach is that enclosed glioma cells in hollow fiber which implanted in mouse brain and attract various kinds of intrinsic brain cells can be clearly compared to those used *in vitro* and *in situ*. When compare to conventional tumor induction, the hollow fiber method allow me to distinguish the influence of cell derived molecules from that of direct cell contact between host CNS cells and transplanted cells. However, the drawback of HF model is that I couldn't monitor the states of cell attraction during different states of tumor progression because glioma cells are entrapped in HF and thus they are not able to form a solid tumor.

## 5.2. Role of microglia in glioma

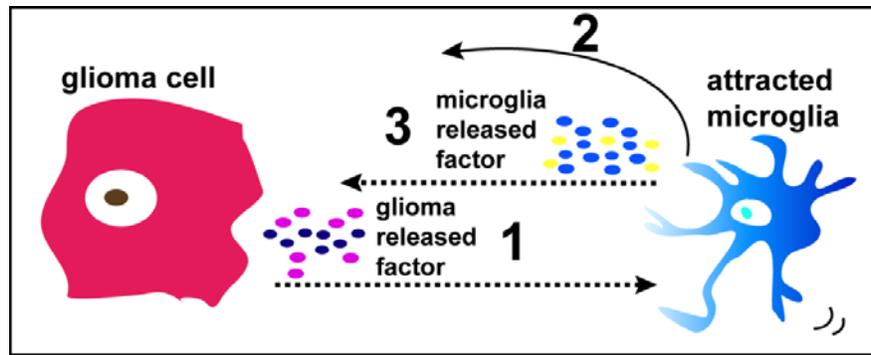
The proliferation of microglial cells in the CNS, a characteristic sign for their activation, is observed during various pathological processes and is a key component of the response to damage including brain tumors (Graeber and Streit 2009). An earlier study showed different proliferative activities at different grades of brain malignancy although the proliferation rate does not solely reflect the proliferation of tumor cells (Klein and Roggendorf 2001). Several lines of *in vitro* studies have depicted that cytokine GM-CSF and IL3 are stimulator of microglia proliferation as shown by  $^3\text{H}$  thymidine incorporation (Ganter, Northoff et al. 1992). IL1 $\beta$  or TNF $\alpha$  also stimulated the proliferation of microglia in mixed glial cultures and pure microglia cultures (Mander, Jekabsone et al. 2006). Recent years a *in vivo* study has also addressed microglia proliferation can be stimulated in neuroimmflamation conditions such as experimental autoimmune encephalomyelitis (EAE) or bacterial infections (Shankaran, Marino et al. 2007).

Glioma cells interact with intrinsic brain cells, in particular microglia/brain macrophages, astrocytes and endogenous neural precursor cells (Charles, Holland et al. 2011). Accompany by growing tumor, cell attraction is found increase surround the glioma. Microglial cells are attracted to glioma and the glioma mass can consist for up to 30 % of microglia/macrophages (Cornejo, Nambi et al. 2010). These tumor associated brain macrophages are a mixed cell population of intrinsic microglia and peripheral monocytes that

entered the CNS through the blood brain barrier (Badie and Schartner 2001). As a brain resident immune cell, they are expected to eliminate the glioma progression. However, when depletion of microglia both *in vitro* (Hussain, Yang et al. 2006) and *in vivo* (Markovic, Vinnakota et al. 2009), impaired tumor growth was found indicating that microglia interact with glioma cells and promote tumor growth (Markovic, Glass et al. 2005). One important mechanism of this glioma-microglia interaction is mediated by the upregulation of the enzyme MT1-MMP in microglia triggered by so far unknown soluble factors released from the glioma cells. MT1-MMP cleaves and thus activates MMP-2, an extracellular matrix protease released from HG-gliomas. This interaction leads to the degradation of the extracellular matrix and further promotes glioma invasion.

Furthermore, glioma recruited microglia/macrophages release many factors, such as chemokines, cytokines, growth factors and proteases, which may directly or indirectly influence tumor progression (Graeber, Scheithauer et al. 2002). It has been shown that GL261 cell migration occurred earlier and faster in the presence of primary microglia or microglia-conditioned medium, which indicated that microglia-released soluble factors can promote glioma migration (Bettinger, Thanos et al. 2002). In addition, recent report pointed out that glioma-infiltrating myeloid cells are the major source of MMP-9, an extracellular matrix protease that promotes glioma angiogenesis (Du, Lu et al. 2008).

Although microglia are attracted to tumor site, they did not appear stimulated to produce pro-inflammatory cytokines (TNF $\alpha$ , IL1, or IL6). In other words, their capacity to activate antitumor effector T cells is not sufficient to initiate anti-tumor responses (Hussain, Yang et al. 2006). In addition, The BTSC (CD133+ like glioma cell) produced colony stimulating factor (CSF-1), transforming growth factor (TGF $\beta$ ), and macrophage inhibitory cytokine 1 (MIC-1), cytokines known to recruit and also polarize the microglia/macrophage to become immunosuppressive via inhibition phagocytosis and secretion of the immunosuppressive cytokines IL10 and TGF $\beta$ 1 (Wu, Wei et al. 2010). In addition, genes characteristic for the alternative and pro-invasive phenotype (*arg-1*, *mt1-mmp*, *cxcl14*) and increased expression of IL-10 and GM-CSF in glioma-bearing CD11b+ cells were found in an experimental murine GL261 glioma model (Gabrusiewicz, Ellert-Miklaszewska et al. 2011). These findings suggested that gliomas recruit microglia, inhibit the immune response and trigger those tumor associated microglia produce tumor survival factors, which facilitates glioma progression and malignancy.



**Fig 5.1. Illustration showing the interaction of tumor cell and microglia in glioma.** Glioma cells secrete various factors involved in infiltration of microglia (1 and 2). Attracted microglia can further secrete factors such as cytokines and chemokines which influence glioma cells (3).

In this study, I have initially found that glioma induction in C57BL/6 mouse brain recruit massive amount of microglia (Fig 1.7). Further using HF approach, microglia accumulation was also observed in adjacent HF with glioma cell encapsulation, indicating microglia-glioma association. In the present study I have also identified GDNF as a factor released from glioma, which acts as a chemoattractant for microglia and promotes glioma growth.

### 5.3. GDNF is a key factor for microglia recruitment in glioma

GDNF was originally identified as a factor released from a glioma cell line and has been identified as growth factor acting on different types of neurons (Lin, Doherty et al. 1993). GDNF is especially a potent survival factor for dopaminergic neurons of the nigrostriatal pathway and is considered as a therapeutic target in Parkinson's disease (Kirik, Georgievska et al. 2004; Lang, Gill et al. 2006). GDNF has been proposed to be chemoattractive to several types of cells such as Schwann cells (Cornejo, Nambi et al.), neuronal precursor cells (Paratcha, Ibanez et al. 2006), and epithelial cells (Tang, Worley et al. 1998) based on *in vitro* and *in vivo* studies; however, a chemotactic effect on microglia by GDNF has not been reported previously. This thesis first presents glioma released factor moderate microglia infiltration. Secondly I confirmed that glioma produce and release GDNF. Forced-expression of GDNF in fibroblast cells attracts microglia while blocking GDNF on glioma cells reduced microglia attraction *in vivo*. Subsequently I used several chemotaxis assays to confirm their chemoattraction phenomenon. Agarose spot assay clearly showed that microglia migrates towards the source of GDNF spot in time and dose dependent manners.

Additionally, GDNF has been found has a positive regulatory effect on rat microglia activities, such as phagocytosis and upregulation of adhesion molecules (Chang, Fang et al. 2006). In my thesis, I found that GDNF promote the proliferation of mouse primary microglia which

implied GDNF has positive regulation on microglia. However, more functional assays for example: phagocytosis assay or cytokine secretion from GDNF stimulated microglia could be done.

Glioma cells are associated with various aggressive tumor behaviors, such as proliferation, chemoattraction, migration and invasion both *in vitro* and *in vivo* (Nakada, Kita et al. 2011). To determine whether GDNF is involved in glioma pathology, the relation between GDNF expression and glioma invasiveness was determined. I first stained mouse brain tissue from experimental malignant glioma and human glioma specimens with anti-GDNF antibody. The staining result showed both human and mouse glioma expressed GDNF. This result consistent with the previous finding in human glioma that GDNF expression is highly abundant in glioma area (Wiesenhofer, Stockhammer et al. 2000). GDNF was implied to promote rat C6 glioma cell migration (Du, Lu et al. 2008). Indeed, when I applied agarose spot assay to test if GDNF has chemotaxis effect on GL261 glioma cells, there was an effect on glioma cell migration. Besides chemotaxis effect, in 2000, Wiesenhofer *et al.* also showed GDNF can be a crucial factor for C6 glioma cell proliferation by antisense approach (Wiesenhofer, Weis et al. 2000). In my study, I could show by cell proliferation assay, the BrdU uptake increased by GL261 cell after adding GDNF in glioma cell culture, which implied GDNF promote glioma cell proliferation. When blocking GDNF in glioma cells, the tumor growth is diminished in a mouse glioma model; however, more sophisticated experiments such as blocking GDNF receptor could be addressed more in the future.

Several lines of studies have shown that knock down of certain genes in glioma cells can impair their invasiveness. For example, knock down of HIF-1 $\alpha$  reduces hypoxia-induced migration of human glioma cells and invasive by interfering number and size of tumor spheres although the tumor size wasn't affected *in vivo* (Mendez, Zavadil et al. 2010). Inhibition of macrophage migration inhibitory factor (MIF) released by glioma cells using specific MIF inhibitors suspends glioma cell tumor growth and migration/invasion (Piette, Deprez et al. 2009). Conversely, the blockade of endogenous CX3CL1 by means of a neutralizing monoclonal antibody markedly delayed tumor cell aggregation and increased their invasiveness (Sciume, Soriani et al. 2010). Furthermore, growth factors known to induce macrophage/microglia proliferation, such as CSF-1, G-CSF and hepatocyte growth factor/ scatter factor (HGF/SF) are also secreted by various gliomas. In the present study I provide evidence that GDNF is a chemoattractant for microglia. I have demonstrated that (1) recombinant GDNF is a chemoattractant for cultured microglial cells as established in 3 different assays, (2) glioma conditioned supernatant from glioma cells with impaired GDNF expression has a lower potency as chemoattractant for microglia as compared to control conditioned medium, (3) glioma cells with down regulated GDNF expression encapsulated into HF and implanted into mouse brain attract less microglia as compared to control glioma

cells, (4) overexpression of GDNF in a fibroblast cell line attracted more microglia as HF with control fibroblasts in our *in vivo* model, (5) orthotopic implantation of GDNF knockdown glioma cells into the mouse brain generate smaller tumors as compared to controls. Importantly, reduced GDNF levels in gliomas improved survival in our immunocompetent, orthotopic implantation model. My data also indicate that GDNF is a major but not the least chemoattractant for microglia/macrophages to HG-gliomas. Other factors like MCP-1 (CCL-2), CX3CL1 or SDF-1 (CXCL-12) may also contribute to microglial attraction to primary brain tumors. However, the role of these factors on the recruitment of microglia towards glioma have only been described *in vitro* (Zhao, Najbauer et al. 2008; Kenig, Alonso et al.; Magge, Malik et al. 2009). I have tried by mass spectrometry method to analyze the protein component which released by glioma cell (GCM). However, this method could not detect any chemokines in GCM due to technical problem. It will be an important task to finding more potential interacting factors between microglia and glioma cells, more thorough experiments are worth to try.

Glioma cells also induce reactive astrogliosis (Le, Besson et al. 2003). This is accompanied by an upregulation of GFAP and a morphological transformation. I observed that glioma encapsulated HF induce astrogliosis. This effect is specific to glioma cells since implanted fibroblast did not trigger such a response. I found, however, that GDNF depletion in glioma cells or overexpression in fibroblast cells do not affect astrogliosis indicating that factors other than GDNF mediate glioma-astrocyte interaction. In CNS, GDNF receptors have been found expressed on both microglia and astrocytes. For example, rat primary microglia culture was tested by RT-PCR, and showed basal weak level of GDNF receptors (Honda, Nakajima et al. 1999). Some stimuli such as lipopolysaccharide (LPS) may directly regulate the expression of GDNF, neurturin and GFR $\alpha$ 1 on microglia, since human microglial express the LPS binding receptor CD14 (Becher and Antel 1996). I also found, GDNF receptors are weakly expressed on naïve microglia but upregulated upon glioma conditioned medium stimulation. Which explain the potential ligand-receptor mechanism of GDNF chemotatic effect on microglia. On the other hand, astrocytes also express GDNF receptors (Wordinger, Lambert et al. 2003). RT-PCR results revealed that the basal level of GDNF receptor expression was already high. Upon treatment of GCM for several hours even up to 4 days, there was no change of level of GDNF receptor expression which might also explain why there was no effect on astrogliosis by GDNF.

Since 2000 Wiesenhofer et al. discovered that GDNF and its receptor GFR $\alpha$ 1 are strongly expressed in human glioma, few researches were carried on. Pre-treatment of glioblastoma cell lines with GDNF, promoted its mitogenic behavior and increase chemoresistance (Ng, Wan et al. 2009). In addition, GDNF enhances the migration of human glioma cell lines (U87

and U251 cells) and increase the expression of matrix metalloproteinase-13 (MMP-13) expression and secretion (Lu, Leung et al. 2010) which implied the aggressiveness of glioma cells. Some studies investigated on the relations between GDNF and GFRs on other tumors, such as pancreatic cancer (Ito, Okada et al. 2005), breast cancer (Esseghir, Todd et al. 2007), colon carcinoma (Qiao, Iwashita et al. 2009). Many studies found GDNF produced a marked effect through binding the special receptor compound (GFR $\alpha$ -I and RET protein) and activating a certain signal transmission pathway. But it is unknown what mechanism GDNF binds these two different receptors by and then what signal transmission pathway is activated. Although GDNF has preference to bind to GFR $\alpha$ 1, it cannot be excluded that it also binds to GFR $\alpha$ 2, GFR $\alpha$ 3, or GFR $\alpha$ 4. Most of the studies mentioned that the GDNF signaling pathway is through first binding to GFR $\alpha$ 1 then form a complex with RET, however, GFR can be signaling independently with RET (Paratcha, Ledda et al. 2001).

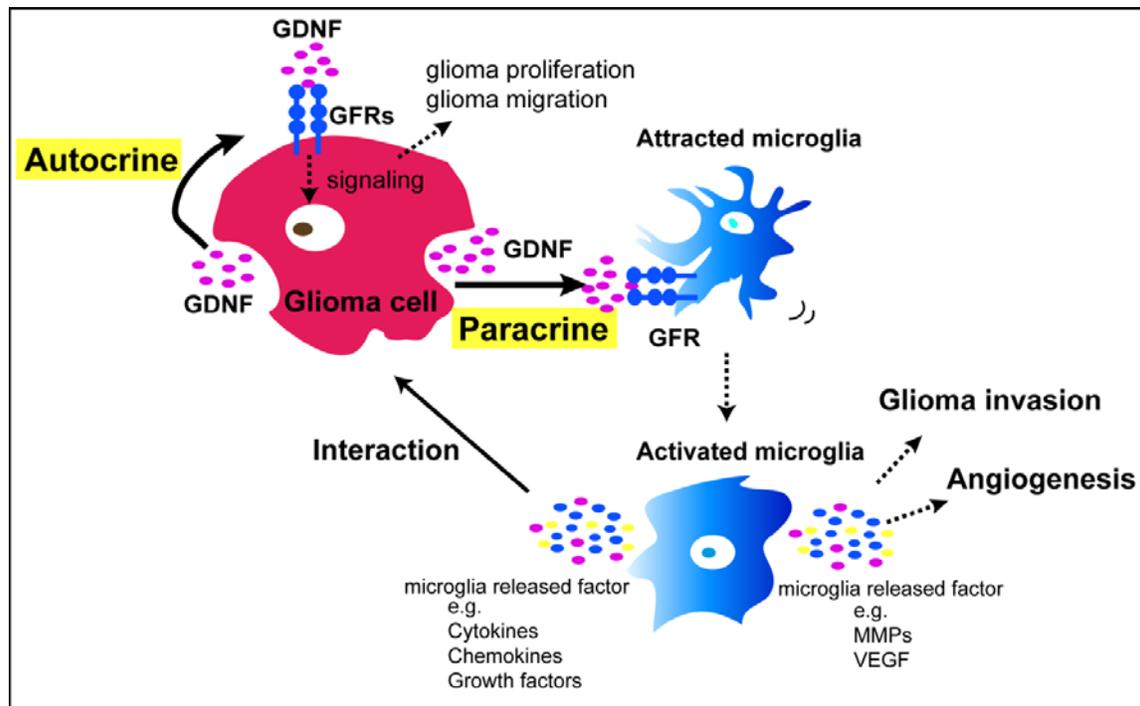
Indeed, when I measured the GFR and RET expression on microglia upon GCM stimulation, there was no change on RET expression. In general the basal level of RET gene expression was very low, can barely detected on microglia. I couldn't detect the level of expression of GFR $\alpha$ 3 and GFR $\alpha$ 4, either.

#### **5.4. GDNF has autocrine and paracrine effect on microglia and glioma cell**

A recent sophisticated study has shown that both cell population dynamics and soluble signaling proteins fulfill the cell-cell communication network and tackle how this network controls glioma progression by observing the *in silico* dynamics of the glioma microenvironment (Wu, Lu et al. 2012). HG glioma cells secrete several factors that promote glioma proliferation and migration, which result in autocrine signaling. Emerging studies have shown evidence that glioma cells use vascular endothelial growth factor (VEGF), a mitogen specific to endothelial cells (Nomura, Yamagishi et al. 1995), SDF-1 (Barbero, Bonavia et al. 2003), glutamate (Lyons, Chung et al. 2007), and catabolite kynurenine (Kyn), an endogenous ligand (Opitz, Litzemberger et al. 2011) as autocrine or paracrine signals to promote cell migration/invasion. In this thesis, I also aimed to discover the factors which serve as a mediator between tumor cells and CNS cells communication.

GDNF was implied to promote glioma cell migration (Song and Moon 2006). Thus I think it could be a potential target. From *in vitro* experiment, I found that upon glioma cell conditioned medium stimulation, GFR mRNA is upregulated in microglia. In the mean time, glioma released GDNF trigger microglia migration. BrdU assay also revealed GDNF can promote microglia proliferation. These results indicate GDNF has paracrine effect on

adjacent microglia. From *in vivo* experiment, I found that depletion of GDNF in glioma can decrease the size of the tumor and prolong the mice survival. My data suggest that glioma invasion is effectively disrupted by inhibiting the GDNF autocrine functional loop with a gene knockdown approach, which indicate GDNF has autocrine effect. Hence, HG glioma cell released GDNF activates GDNF receptors either on the same or adjacent cells, hence acting in either autocrine or paracrine fashion.



**Fig 5.2. A proposed model of GDNF paracrine and autocrine effects in glioma.** Glioma cells secrete various autocrine motility factors including GDNF that maintain the process of invasion through attracting more microglia. GDNF worked on both glioma cells and microglia. Those cells further activated and form a paracrine and autocrine loop.

## 5.5. Has GDNF a potential clinical implication for glioma treatment?

The main stream of clinical usage of GDNF has focused on Parkinson's disease. Chronic and direct GDNF infusion into brain has been found promotes structural and functional recovery in Parkinson's disease (Grondin, Zhang et al. 2002; Gill, Patel et al. 2003). By far, the effect of GDNF therapy is still controversy due to the safety and delivery problem (Yasuhara, Shingo et al. 2007). On the contrary of GDNF bust, in the tumor context, deletion of GDNF is the potential target. Because GDNF promotes the progression of pancreatic cancer, researcher has tried to block the GDNF signaling pathway by inhibitors (Liu, Li et al. 2012). Some other extended study has targeted the GDNF signaling partner RET by small

molecule inhibitors of RET kinase activity for treating thyroid cancer (Wells and Santoro 2009; Mologni 2010). However, to my knowledge there is no study shown blocking GDNF in glioma. The only clue from the Affymetrix data, GDNF gene expression in the human GBM samples doesn't affect the survival rate (<https://caintegrator.nci.nih.gov/rembrandt>).

## 5.6. CCL5 and glioma

RANTES is a basic 8-kDa polypeptide of the C–C chemokine subfamily known with strong chemoattractant activity for T lymphocytes and monocytes/macrophages. In CNS, CCL5 is produced by astrocytes, activated endothelium or possibly other kinds of glial cells to recruit T lymphocytes from the peripheral circulation to perivascular regions within the brain (Hesselgesser and Horuk 1999). It has been found that one of its major receptor CCR5 is not only essential for chemotaxis of leukocytes but also has been shown to be a key co-receptor for HIV-1 infection in the CNS.

The glioma microenvironment often includes many elements which regulates stromal cell infiltration, however, CCL5 alone has not yet been extensively discussed. Limited studies have shown that RANTES was induced in a dose- and time-dependent manner by measles virus infection in a human astrocytoma cell line, U373 (Noe, Cenciarelli et al. 1999). Moreover, astrocytoma cells showed upregulated CCL5 upon co-culture with activated peripheral monocytes (Jehs, Faber et al. 2011), which implied the potential interaction between glioma cell and tumor associated stromal cells. A more recent study has found that using medulloblastoma as a model; T lymphocytes may be attracted to tumor by CCL5-fendothelial cells crosstalk (Salsman, Chow et al. 2011).

Recent years, a new concept for glioma has brought up the attention, which is, the association between human cytomegalovirus (HCMV) infection and because of the presence of this virus in glioma tissue. HCMV is a ubiquitous herpesvirus that exhibits tropism for glial cells and has been shown to transform cells in vitro. Thus, HCMV may be a hint for development of gliomas (Scheurer, Bondy et al. 2008). Interestingly, one study has explored an oncogenic role for US28 (G protein–coupled receptor like protein) which encoded by HCMV. The invasive phenotype can be enhanced by exposure to CCL5, which is a ligand of US28. Taken together all those findings from literature, we know that CCL5 contribute to glioma progression and suggest that targeting CCL5 may provide therapeutic benefits in GBM treatment (Soroceanu, Matlaf et al. 2011).

## 6. Summary

High grade gliomas (HG-glioma) are the most common primary brain tumors. Their malignancy depends on complex crosstalk between different cell types in the CNS. Our group has previously shown that endogenous neural progenitor cells (NPC) contribute to glioma regression; conversely, brain resident immune cells (microglia) infiltrate gliomas and contribute to their growth progression. Thus, understanding the component of glioma microenvironment is a crucial goal for glioma therapy.

In this thesis, I aimed to identify soluble factors secreted by glioma cells which could mediate glioma-CNS cells interaction. To identify these factors, I established a protocol to encapsulate glioma cells into a hollow fiber (HF) which allows the passage of diffusible molecules, but not cells. By utilizing the HF model, I first demonstrated the infiltration of microglia and NPC although glioma cells were entrapped in isolated fiber while NG2 cells were not affected. Astrocytes surrounding the HF showed increased GFAP immunoreactivity, a marker of astrogliosis. Furthermore, human glioma cells can also be encapsulated in HF and were similarly effective in attracting microglial cells or increasing astrogliosis in mouse brain. I showed here for the first time that GDNF is a chemo-attractant for microglia. Data from various migration assays and *in vivo* test suggest that GDNF, a known neurotrophic factor for neurons, which is released by glioma cells, has chemotaxis effect on microglia. Further reducing GDNF secretion from glioma cells by siRNA approach, the recruitment of microglia toward glioma HF was significantly diminished while astrogliosis was not affected. Conversely, forced secretion of GDNF from fibroblast cells increased microglia infiltration. All together, I found that GDNF plays a pivotal role in microglia infiltration but not astrocytes. Finally, to confirm if attenuation of microglia migration can further influence glioma growth, stable GDNF knockdown in glioma by shRNA method was conducted. When implanting GDNF knockdown glioma cells into mouse brain, smaller tumor was formed and the survival of mice improved.

In conclusion, I identified GDNF as a HG-glioma released factor which specifically leads to microglial attraction and thereby serves a tumor promoting role. This study supports the idea that microglia play an important role in tumor growth, invasion and progression and thus can become a novel target for therapeutic strategies.

## 7. Zusammenfassung

Das Glioblastoma multiforme (GBM) zählt zu den häufigsten primären Hirntumoren, deren Malignität von komplexen Wechselwirkungen zwischen verschiedenen Zelltypen des Zentralen Nervensystems abhängt. In vorangegangenen Arbeiten konnte unsere Gruppe zeigen, dass Endogene Neuronale Progenitorzellen zur Gliom Regression beiträgt. Im Gegensatz dazu infiltrieren Immunzellen des Gehirns, sogenannte Gliazellen, Gliome und tragen zur Tumorprogression bei. Daher ist es unerlässlich die Gliom-Umgebung und deren Wechselwirkungen genauer zu verstehen um Gliom-Therapien zu verbessern.

Das Ziel dieser vorgelegten Doktorarbeit ist es, Faktoren zu identifizieren, die von Gliom-Zellen sezerniert werden und eine Wechselwirkung zwischen Gliom-Zellen und Zellen des Zentralen Nervensystems vermitteln. Um diese Faktoren zu identifizieren, habe ich ein Protokoll etabliert, bei dem Gliom-Zellen in eine Hohlfaser (HF) eingekapselt werden. Die HF erlaubt den Austausch löslicher Stoffe, verhindert aber den Austritt von Zellen und somit einen direkten Zell-Kontakt. Mit der Anwendung des HF Modells konnte ich zeigen, dass eingekapselte und ins Mausgehirn transplantierte Gliom-Zellen zu einer Infiltrierung durch Mikroglia und Neuronale Progenitorzellen führen. Eine Infiltrierung durch NG2 Zellen wurde nicht beobachtet. Astrozyten die die transplantierte HF umgeben wiesen eine erhöhte GFAP Immunoreaktivität auf, einem Marker für Astrogliose. Außerdem führte die Einkapselung von humanen Gliom-Zellen im gleichen Maße zur Infiltrierung durch Mikroglia und Astrogliose führten. Im Weiteren konnte ich erstmals zeigen, dass GDNF ein Chemotaktischer Faktor für Mikroglia ist. Verschiedene Migrations-Assay und *in vivo* Experimente haben verdeutlicht, dass GDNF, ein bekannter neurotropischer Faktor für Neuronen der von Gliom-Zellen sezerniert wird, einen Chemotaktischen Einfluss auf Mikroglia hat. Die Reduzierung der GDNF Sekretion von Gliom-Zellen durch siRNAs führte zur Reduzierung der Mikroglia-Infiltrierung, wobei die Astrogliose unverändert blieb. Im Gegensatz dazu führte die Überexpression von GDNF in Fibroblasten zu einer erhöhten Infiltrierung durch Mikroglia. In dieser Studie konnte ich zeigen, dass GDNF eine zentrale Rolle bei der Infiltrierung durch Mikroglia spielt, die Astrogliose jedoch nicht beeinflusst. Um zuletzt zu zeigen, dass die verminderte Infiltrierung durch Mikroglia das Gliom-Wachstum beeinflusst, wurde die GDNF Sekretion in Gliom-Zellen mittels shRNA stabil vermindert. Die Implantation von GDNF Knockdown Gliom-Zellen ins Mausgehirn, resultierte in kleineren Tumoren und das Überleben der Maus wurde verlängert.

Zusammenfassend bleibt festzuhalten, dass ich GDNF als Gliom-sezernierten Faktor identifiziert habe, der spezifisch zur Attraktion von Mikroglia führt und somit zum Tumorwachstum beiträgt. Diese Arbeit verdeutlicht die Rolle von Mikroglia beim Wachstum, der Invasion und der Progression von Tumoren und stellt daher einen neuartigen potentiellen Therapieansatz da.

## 8. References

- Aboody, K. S., A. Brown, et al. (2000). "Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas." Proc Natl Acad Sci U S A **97**(23): 12846-51.
- Airaksinen, M. S. and M. Saarma (2002). "The GDNF family: signalling, biological functions and therapeutic value." Nat Rev Neurosci **3**(5): 383-94.
- Al-Mayhany, M. T., R. Grenfell, et al. (2011). "NG2 expression in glioblastoma identifies an actively proliferating population with an aggressive molecular signature." Neuro Oncol **13**(8): 830-45.
- Anderson, C. M. and M. Nedergaard (2003). "Astrocyte-mediated control of cerebral microcirculation." Trends Neurosci **26**(7): 340-4; author reply 344-5.
- Badie, B. and J. Schartner (2001). "Role of microglia in glioma biology." Microsc Res Tech **54**(2): 106-13.
- Badie, B., J. Schartner, et al. (1999). "In vitro modulation of microglia motility by glioma cells is mediated by hepatocyte growth factor/scatter factor." Neurosurgery **44**(5): 1077-82; discussion 1082-3.
- Badie, B. and J. M. Schartner (2000). "Flow cytometric characterization of tumor-associated macrophages in experimental gliomas." Neurosurgery **46**(4): 957-61; discussion 961-2.
- Barbero, S., R. Bonavia, et al. (2003). "Stromal cell-derived factor 1alpha stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt." Cancer Res **63**(8): 1969-74.
- Barnes, D. A., M. Huston, et al. (1996). "Induction of RANTES expression by astrocytes and astrocytoma cell lines." J Neuroimmunol **71**(1-2): 207-14.
- Barres, B. A. (2008). "The mystery and magic of glia: a perspective on their roles in health and disease." Neuron **60**(3): 430-40.
- Becher, B. and J. P. Antel (1996). "Comparison of phenotypic and functional properties of immediately ex vivo and cultured human adult microglia." Glia **18**(1): 1-10.
- Becher, O. J., D. Hambardzumyan, et al. (2008). "Gli activity correlates with tumor grade in platelet-derived growth factor-induced gliomas." Cancer Res **68**(7): 2241-9.
- Bechmann, I. and R. Nitsch (2001). "Plasticity following lesion: help and harm from the immune system." Restor Neurol Neurosci **19**(3-4): 189-98.
- Belmadani, A., P. B. Tran, et al. (2006). "Chemokines regulate the migration of neural progenitors to sites of neuroinflammation." J Neurosci **26**(12): 3182-91.
- Belperio, J. A., M. P. Keane, et al. (2000). "CXC chemokines in angiogenesis." J Leukoc Biol **68**(1): 1-8.
- Bettinger, I., S. Thanos, et al. (2002). "Microglia promote glioma migration." Acta Neuropathol **103**(4): 351-5.
- Boscia, F., C. L. Esposito, et al. (2009). "GDNF selectively induces microglial activation and neuronal survival in CA1/CA3 hippocampal regions exposed to NMDA insult through Ret/ERK signalling." PLoS One **4**(8): e6486.
- Brenner, A. V., M. A. Butler, et al. (2007). "Single-nucleotide polymorphisms in selected cytokine genes and risk of adult glioma." Carcinogenesis **28**(12): 2543-7.
- Bruni, J. E. (1998). "Ependymal development, proliferation, and functions: a review." Microsc Res Tech **41**(1): 2-13.

- Carrillo-de Sauvage, M. A., A. Gomez, et al. (2012). "CCL2-expressing astrocytes mediate the extravasation of T lymphocytes in the brain. Evidence from patients with glioma and experimental models in vivo." *PLoS One* **7**(2): e30762.
- Carson, M. J. (2002). "Microglia as liaisons between the immune and central nervous systems: functional implications for multiple sclerosis." *Glia* **40**(2): 218-31.
- Chang, Y. P., K. M. Fang, et al. (2006). "Regulation of microglial activities by glial cell line derived neurotrophic factor." *J Cell Biochem* **97**(3): 501-11.
- Charles, N. A., E. C. Holland, et al. (2011). "The brain tumor microenvironment." *Glia* **59**(8): 1169-80.
- Chick, W. L., J. J. Perna, et al. (1977). "Artificial pancreas using living beta cells: effects on glucose homeostasis in diabetic rats." *Science* **197**(4305): 780-2.
- Chirasani, S. R., A. Sternjak, et al. (2010). "Bone morphogenetic protein-7 release from endogenous neural precursor cells suppresses the tumorigenicity of stem-like glioblastoma cells." *Brain* **133**(Pt 7): 1961-72.
- Coniglio, S. J., E. Eugenin, et al. (2012). "Microglial Stimulation of Glioblastoma Invasion Involves Epidermal Growth Factor Receptor (EGFR) and Colony Stimulating Factor 1 Receptor (CSF-1R) Signaling." *Mol Med* **18**(1): 519-27.
- Cornejo, M., D. Nambi, et al. "Effect of NRG1, GDNF, EGF and NGF in the migration of a Schwann cell precursor line." *Neurochem Res* **35**(10): 1643-51.
- Cornejo, M., D. Nambi, et al. (2010). "Effect of NRG1, GDNF, EGF and NGF in the migration of a Schwann cell precursor line." *Neurochem Res* **35**(10): 1643-51.
- Davalos, D., J. Grutzendler, et al. (2005). "ATP mediates rapid microglial response to local brain injury in vivo." *Nat Neurosci* **8**(6): 752-8.
- de la Iglesia, N., G. Konopka, et al. (2008). "Deregulation of a STAT3-interleukin 8 signaling pathway promotes human glioblastoma cell proliferation and invasiveness." *J Neurosci* **28**(23): 5870-8.
- Del Bigio, M. R. (1995). "The ependyma: a protective barrier between brain and cerebrospinal fluid." *Glia* **14**(1): 1-13.
- Dhandapani, K. M., M. M. Khan, et al. (2007). "Induction of transforming growth factor-beta1 by basic fibroblast growth factor in rat C6 glioma cells and astrocytes is mediated by MEK/ERK signaling and AP-1 activation." *J Neurosci Res* **85**(5): 1033-45.
- Diers-Fenger, M., F. Kirchhoff, et al. (2001). "AN2/NG2 protein-expressing glial progenitor cells in the murine CNS: isolation, differentiation, and association with radial glia." *Glia* **34**(3): 213-28.
- Doetsch, F. (2003). "The glial identity of neural stem cells." *Nat Neurosci* **6**(11): 1127-34.
- Domanska, U. M., R. C. Kruizinga, et al. (2010). "The chemokine network, a newly discovered target in high grade gliomas." *Crit Rev Oncol Hematol* **79**(2): 154-63.
- Du, R., K. V. Lu, et al. (2008). "HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion." *Cancer Cell* **13**(3): 206-20.
- Dudanova, I., G. Gatto, et al. (2010). "GDNF acts as a chemoattractant to support ephrinA-induced repulsion of limb motor axons." *Curr Biol* **20**(23): 2150-6.
- Eddleston, M. and L. Mucke (1993). "Molecular profile of reactive astrocytes--implications for their role in neurologic disease." *Neuroscience* **54**(1): 15-36.
- Edwards, L. A., K. Woolard, et al. (2011). "Effect of brain- and tumor-derived connective tissue growth factor on glioma invasion." *J Natl Cancer Inst* **103**(15): 1162-78.
- Ehteshami, M., J. A. Winston, et al. (2006). "CXCR4 expression mediates glioma cell invasiveness." *Oncogene* **25**(19): 2801-6.

- Emerich, D. F. and H. C. Salzbeg (2001). "Update on immunoisolation cell therapy for CNS diseases." Cell Transplant **10**(1): 3-24.
- Emsley, J. G., B. D. Mitchell, et al. (2005). "Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells." Prog Neurobiol **75**(5): 321-41.
- Esseghir, S., S. K. Todd, et al. (2007). "A role for glial cell derived neurotrophic factor induced expression by inflammatory cytokines and RET/GFR alpha 1 receptor up-regulation in breast cancer." Cancer Res **67**(24): 11732-41.
- Farber, K. and H. Kettenmann (2006). "Purinergic signaling and microglia." Pflugers Arch **452**(5): 615-21.
- Farina, C., F. Aloisi, et al. (2007). "Astrocytes are active players in cerebral innate immunity." Trends Immunol **28**(3): 138-45.
- Fasano, C. A., T. N. Phoenix, et al. (2009). "Bmi-1 cooperates with Foxg1 to maintain neural stem cell self-renewal in the forebrain." Genes Dev **23**(5): 561-74.
- Flynn, G., S. Maru, et al. (2003). "Regulation of chemokine receptor expression in human microglia and astrocytes." J Neuroimmunol **136**(1-2): 84-93.
- Franklin, G. P. a. K. B. J. (2007). The Mouse Brain in Stereotaxic Coordinates, Third Edition, Academic Press.
- Furnari, F. B., T. Fenton, et al. (2007). "Malignant astrocytic glioma: genetics, biology, and paths to treatment." Genes Dev **21**(21): 2683-710.
- Gabrusiewicz, K., A. Ellert-Miklaszewska, et al. (2011). "Characteristics of the alternative phenotype of microglia/macrophages and its modulation in experimental gliomas." PLoS One **6**(8): e23902.
- Ganter, S., H. Northoff, et al. (1992). "Growth control of cultured microglia." J Neurosci Res **33**(2): 218-30.
- Geranmayeh, F., B. W. Scheithauer, et al. (2007). "Microglia in gemistocytic astrocytomas." Neurosurgery **60**(1): 159-66; discussion 166.
- Ghorpade, A., H. Gendelman, et al. (2008). Macrophage, microglia and dendritic cells. Neuroimmune Pharmacology. T. I. Howard E. Gendelman, Springer-Verlag New York Inc: 89-104.
- Ghosh, A. and S. Chaudhuri (2010). "Microglial action in glioma: a boon turns bane." Immunol Lett **131**(1): 3-9.
- Gil, Z., O. Cavel, et al. (2010). "Paracrine regulation of pancreatic cancer cell invasion by peripheral nerves." J Natl Cancer Inst **102**(2): 107-18.
- Gill, S. S., N. K. Patel, et al. (2003). "Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease." Nat Med **9**(5): 589-95.
- Glass, R., M. Synowitz, et al. (2005). "Glioblastoma-induced attraction of endogenous neural precursor cells is associated with improved survival." J Neurosci **25**(10): 2637-46.
- Graeber, M. B., B. W. Scheithauer, et al. (2002). "Microglia in brain tumors." Glia **40**(2): 252-9.
- Graeber, M. B. and W. J. Streit (2009). "Microglia: biology and pathology." Acta Neuropathol **119**(1): 89-105.
- Grondin, R., Z. Zhang, et al. (2002). "Chronic, controlled GDNF infusion promotes structural and functional recovery in advanced parkinsonian monkeys." Brain **125**(Pt 10): 2191-201.
- Halks-Miller, M., M. L. Schroeder, et al. (2003). "CCR1 is an early and specific marker of Alzheimer's disease." Ann Neurol **54**(5): 638-46.
- Han, Q., W. Sun, et al. (2009). "Linear ordered collagen scaffolds loaded with collagen-binding brain-derived neurotrophic factor improve the recovery of spinal cord injury in rats." Tissue Eng Part A **15**(10): 2927-35.

- Hanisch, U. K. and H. Kettenmann (2007). "Microglia: active sensor and versatile effector cells in the normal and pathologic brain." Nat Neurosci **10**(11): 1387-94.
- Hesselgesser, J. and R. Horuk (1999). "Chemokine and chemokine receptor expression in the central nervous system." J Neurovirol **5**(1): 13-26.
- Hoelzinger, D. B., T. Demuth, et al. (2007). "Autocrine factors that sustain glioma invasion and paracrine biology in the brain microenvironment." J Natl Cancer Inst **99**(21): 1583-93.
- Honda, S., K. Nakajima, et al. (1999). "Rat primary cultured microglia express glial cell line-derived neurotrophic factor receptors." Neurosci Lett **275**(3): 203-6.
- Hong, T. M., L. J. Teng, et al. (2009). "Induced interleukin-8 expression in gliomas by tumor-associated macrophages." J Neurooncol **93**(3): 289-301.
- Hussain, S. F., D. Yang, et al. (2006). "The role of human glioma-infiltrating microglia/macrophages in mediating antitumor immune responses." Neuro Oncol **8**(3): 261-79.
- Ito, Y., Y. Okada, et al. (2005). "Expression of glial cell line-derived neurotrophic factor family members and their receptors in pancreatic cancers." Surgery **138**(4): 788-94.
- Iwahashi, N., T. Nagasaka, et al. (2002). "Expression of glial cell line-derived neurotrophic factor correlates with perineural invasion of bile duct carcinoma." Cancer **94**(1): 167-74.
- Jehs, T., C. Faber, et al. (2011). "Astrocytoma cells upregulate expression of pro-inflammatory cytokines after co-culture with activated peripheral blood mononuclear cells." APMIS **119**(8): 551-61.
- Jessen, K. R. (2004). "Glial cells." Int J Biochem Cell Biol **36**(10): 1861-7.
- Karram, K., S. Goebbels, et al. (2008). "NG2-expressing cells in the nervous system revealed by the NG2-EYFP-knockin mouse." Genesis **46**(12): 743-57.
- Kenig, S., M. B. Alonso, et al. "Glioblastoma and endothelial cells cross-talk, mediated by SDF-1, enhances tumour invasion and endothelial proliferation by increasing expression of cathepsins B, S, and MMP-9." Cancer Lett **289**(1): 53-61.
- Kenig, S., M. B. Alonso, et al. (2009). "Glioblastoma and endothelial cells cross-talk, mediated by SDF-1, enhances tumour invasion and endothelial proliferation by increasing expression of cathepsins B, S, and MMP-9." Cancer Lett **289**(1): 53-61.
- Kessenbrock, K., V. Plaks, et al. (2010). "Matrix metalloproteinases: regulators of the tumor microenvironment." Cell **141**(1): 52-67.
- Kettenmann, H., U. K. Hanisch, et al. "Physiology of microglia." Physiol Rev **91**(2): 461-553.
- Kim, Y. T., R. Hitchcock, et al. (2005). "A cell encapsulation device for studying soluble factor release from cells transplanted in the rat brain." J Control Release **102**(1): 101-11.
- Kirik, D., B. Georgievska, et al. (2004). "Localized striatal delivery of GDNF as a treatment for Parkinson disease." Nat Neurosci **7**(2): 105-10.
- Klein, R. and W. Roggendorf (2001). "Increased microglia proliferation separates pilocytic astrocytomas from diffuse astrocytomas: a double labeling study." Acta Neuropathol **101**(3): 245-8.
- Koelsch, A., Y. Feng, et al. (2010). "Transgene-mediated GDNF expression enhances synaptic connectivity and GABA transmission to improve functional outcome after spinal cord contusion." J Neurochem **113**(1): 143-52.
- Kolodziejczyk, K., A. S. Saab, et al. (2010). "Why do oligodendrocyte lineage cells express glutamate receptors?" F1000 Biol Rep **2**: 57.
- Komohara, Y., K. Ohnishi, et al. (2008). "Possible involvement of the M2 anti-inflammatory macrophage phenotype in growth of human gliomas." J Pathol **216**(1): 15-24.

- Kouno, J., H. Nagai, et al. (2004). "Up-regulation of CC chemokine, CCL3L1, and receptors, CCR3, CCR5 in human glioblastoma that promotes cell growth." J Neurooncol **70**(3): 301-7.
- Kreutzberg, G. W. (1996). "Microglia: a sensor for pathological events in the CNS." Trends Neurosci **19**(8): 312-8.
- Krishnamurthy, N. V. and B. Gimi (2011). "Encapsulated cell grafts to treat cellular deficiencies and dysfunction." Crit Rev Biomed Eng **39**(6): 473-91.
- Kuchler, S., M. N. Graff, et al. (1994). "Mannose dependent tightening of the rat ependymal cell barrier. In vivo and in vitro study using neoglycoproteins." Neurochem Int **24**(1): 43-55.
- Lang, A. E., S. Gill, et al. (2006). "Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease." Ann Neurol **59**(3): 459-66.
- Lauffenburger, D. A. and A. F. Horwitz (1996). "Cell migration: a physically integrated molecular process." Cell **84**(3): 359-69.
- Le, D. M., A. Besson, et al. (2003). "Exploitation of astrocytes by glioma cells to facilitate invasiveness: a mechanism involving matrix metalloproteinase-2 and the urokinase-type plasminogen activator-plasmin cascade." J Neurosci **23**(10): 4034-43.
- Lee, G. A., C. H. Lin, et al. (2004). "Microglia-derived glial cell line-derived neurotrophic factor could protect Sprague-Dawley rat astrocyte from in vitro ischemia-induced damage." Neurosci Lett **356**(2): 111-4.
- Leong, S. Y. and A. M. Turnley (2011). "Regulation of adult neural precursor cell migration." Neurochem Int **59**(3): 382-93.
- Leung, S. Y., M. P. Wong, et al. (1997). "Monocyte chemoattractant protein-1 expression and macrophage infiltration in gliomas." Acta Neuropathol **93**(5): 518-27.
- Li, R. H., S. Williams, et al. (1999). "Dose control with cell lines used for encapsulated cell therapy." Tissue Eng **5**(5): 453-66.
- Li, Z., H. Wang, et al. (2009). "Turning cancer stem cells inside out: an exploration of glioma stem cell signaling pathways." J Biol Chem **284**(25): 16705-9.
- Lin, J. H., T. Takano, et al. (2002). "Connexin 43 enhances the adhesivity and mediates the invasion of malignant glioma cells." J Neurosci **22**(11): 4302-11.
- Lin, L. F., D. H. Doherty, et al. (1993). "GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons." Science **260**(5111): 1130-2.
- Liu, H., X. Li, et al. (2012). "Role of glial cell line-derived neurotrophic factor in perineural invasion of pancreatic cancer." Biochim Biophys Acta **1826**(1): 112-20.
- Locatelli, M., L. Boiocchi, et al. (2010). "Human glioma tumors express high levels of the chemokine receptor CX3CR1." Eur Cytokine Netw **21**(1): 27-33.
- Long, J. M., A. N. Kalehua, et al. (1998). "Stereological estimation of total microglia number in mouse hippocampus." J Neurosci Methods **84**(1-2): 101-8.
- Lu, D. Y., Y. M. Leung, et al. (2010). "Glial cell line-derived neurotrophic factor induces cell migration and matrix metalloproteinase-13 expression in glioma cells." Biochem Pharmacol **80**(8): 1201-9.
- Lyck, L., I. D. Santamaria, et al. (2009). "An empirical analysis of the precision of estimating the numbers of neurons and glia in human neocortex using a fractionator-design with sub-sampling." J Neurosci Methods **182**(2): 143-56.
- Lynoe, N., M. Sandlund, et al. (1991). "Informed consent: study of quality of information given to participants in a clinical trial." BMJ **303**(6803): 610-3.
- Lyons, S. A., W. J. Chung, et al. (2007). "Autocrine glutamate signaling promotes glioma cell invasion." Cancer Res **67**(19): 9463-71.

- Lyons, S. A. and H. Kettenmann (1998). "Oligodendrocytes and microglia are selectively vulnerable to combined hypoxia and hypoglycemia injury in vitro." J Cereb Blood Flow Metab **18**(5): 521-30.
- Madinier, A., N. Bertrand, et al. (2009). "Microglial involvement in neuroplastic changes following focal brain ischemia in rats." PLoS One **4**(12): e8101.
- Magge, S. N., S. Z. Malik, et al. (2009). "Role of monocyte chemoattractant protein-1 (MCP-1/CCL2) in migration of neural progenitor cells toward glial tumors." J Neurosci Res **87**(7): 1547-55.
- Mander, P. K., A. Jekabsons, et al. (2006). "Microglia proliferation is regulated by hydrogen peroxide from NADPH oxidase." J Immunol **176**(2): 1046-52.
- Markovic, D. S., R. Glass, et al. (2005). "Microglia stimulate the invasiveness of glioma cells by increasing the activity of metalloprotease-2." J Neuropathol Exp Neurol **64**(9): 754-62.
- Markovic, D. S., K. Vinnakota, et al. (2009). "Gliomas induce and exploit microglial MT1-MMP expression for tumor expansion." Proc Natl Acad Sci U S A **106**(30): 12530-5.
- Martino, G. and S. Pluchino (2006). "The therapeutic potential of neural stem cells." Nat Rev Neurosci **7**(5): 395-406.
- Martino, G. and S. Pluchino (2007). "Neural stem cells: guardians of the brain." Nat Cell Biol **9**(9): 1031-4.
- Mendez, O., J. Zavadil, et al. (2010). "Knock down of HIF-1alpha in glioma cells reduces migration in vitro and invasion in vivo and impairs their ability to form tumor spheres." Mol Cancer **9**: 133.
- Miwa, T., S. Furukawa, et al. (1997). "Lipopolysaccharide enhances synthesis of brain-derived neurotrophic factor in cultured rat microglia." J Neurosci Res **50**(6): 1023-9.
- Mologni, L. (2010). "Development of RET kinase inhibitors for targeted cancer therapy." Curr Med Chem **18**(2): 162-75.
- Murdoch, C. and A. Finn (2000). "Chemokine receptors and their role in inflammation and infectious diseases." Blood **95**(10): 3032-43.
- Nakada, M., D. Kita, et al. (2011). "Aberrant Signaling Pathways in Glioma." Cancers **3**: 3242-3278.
- Ng, W. H., G. Q. Wan, et al. (2009). "Glial cell-line derived neurotrophic factor (GDNF) family of ligands confer chemoresistance in a ligand-specific fashion in malignant gliomas." J Clin Neurosci **16**(3): 427-36.
- Nimmerjahn, A., F. Kirchhoff, et al. (2005). "Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo." Science **308**(5726): 1314-8.
- Noe, K. H., C. Cenciarelli, et al. (1999). "Requirements for measles virus induction of RANTES chemokine in human astrocytoma-derived U373 cells." J Virol **73**(4): 3117-24.
- Nolte, C., T. Moller, et al. (1996). "Complement 5a controls motility of murine microglial cells in vitro via activation of an inhibitory G-protein and the rearrangement of the actin cytoskeleton." Neuroscience **73**(4): 1091-107.
- Nomura, M., S. Yamagishi, et al. (1995). "Possible participation of autocrine and paracrine vascular endothelial growth factors in hypoxia-induced proliferation of endothelial cells and pericytes." J Biol Chem **270**(47): 28316-24.
- Okada, M., M. Saio, et al. (2009). "Tumor-associated macrophage/microglia infiltration in human gliomas is correlated with MCP-3, but not MCP-1." Int J Oncol **34**(6): 1621-7.
- Opitz, C. A., U. M. Litztenburger, et al. (2011). "An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor." Nature **478**(7368): 197-203.

- Ozerdem, U., K. A. Grako, et al. (2001). "NG2 proteoglycan is expressed exclusively by mural cells during vascular morphogenesis." *Dev Dyn* **222**(2): 218-27.
- Paratcha, G., C. F. Ibanez, et al. (2006). "GDNF is a chemoattractant factor for neuronal precursor cells in the rostral migratory stream." *Mol Cell Neurosci* **31**(3): 505-14.
- Paratcha, G., F. Ledda, et al. (2001). "Released GFRalpha1 potentiates downstream signaling, neuronal survival, and differentiation via a novel mechanism of recruitment of c-Ret to lipid rafts." *Neuron* **29**(1): 171-84.
- Parney, I. F., J. S. Waldron, et al. (2009). "Flow cytometry and in vitro analysis of human glioma-associated macrophages. Laboratory investigation." *J Neurosurg* **110**(3): 572-82.
- Pelvig, D. P., H. Pakkenberg, et al. (2008). "Neocortical glial cell numbers in human brains." *Neurobiol Aging* **29**(11): 1754-62.
- Peters, A., K. Josephson, et al. (1991). "Effects of aging on the neuroglial cells and pericytes within area 17 of the rhesus monkey cerebral cortex." *Anat Rec* **229**(3): 384-98.
- Pham, K., D. Luo, et al. (2012). "CCL5, CCR1 and CCR5 in murine glioblastoma: Immune cell infiltration and survival rates are not dependent on individual expression of either CCR1 or CCR5." *J Neuroimmunol* **246**(1-2): 10-7.
- Piette, C., M. Deprez, et al. (2009). "The dexamethasone-induced inhibition of proliferation, migration, and invasion in glioma cell lines is antagonized by macrophage migration inhibitory factor (MIF) and can be enhanced by specific MIF inhibitors." *J Biol Chem* **284**(47): 32483-92.
- Platten, M., A. Kretz, et al. (2003). "Monocyte chemoattractant protein-1 increases microglial infiltration and aggressiveness of gliomas." *Ann Neurol* **54**(3): 388-92.
- Qiao, S., T. Iwashita, et al. (2009). "Increased expression of glial cell line-derived neurotrophic factor and neurturin in a case of colon adenocarcinoma associated with diffuse ganglioneuromatosis." *Clin Neuropathol* **28**(2): 105-12.
- Ransohoff, R. M. and V. H. Perry (2009). "Microglial physiology: unique stimuli, specialized responses." *Annu Rev Immunol* **27**: 119-45.
- Read, T. A., D. R. Sorensen, et al. (2001). "Local endostatin treatment of gliomas administered by microencapsulated producer cells." *Nat Biotechnol* **19**(1): 29-34.
- Richardson, W. D., K. M. Young, et al. (2011). "NG2-glia as multipotent neural stem cells: fact or fantasy?" *Neuron* **70**(4): 661-73.
- Rock, R. B., G. Gekker, et al. (2004). "Role of microglia in central nervous system infections." *Clin Microbiol Rev* **17**(4): 942-64, table of contents.
- Rostene, W., A. Guyon, et al. (2010). "Chemokines and chemokine receptors: new actors in neuroendocrine regulations." *Front Neuroendocrinol* **32**(1): 10-24.
- Saijo, K. and C. K. Glass (2011). "Microglial cell origin and phenotypes in health and disease." *Nat Rev Immunol* **11**(11): 775-87.
- Salimi, K., K. V. Moser, et al. (2003). "GDNF and TGF-beta1 promote cell survival in serum-free cultures of primary rat microglia." *Cell Tissue Res* **312**(1): 135-9.
- Salsman, V. S., K. K. Chow, et al. (2011). "Crosstalk between medulloblastoma cells and endothelium triggers a strong chemotactic signal recruiting T lymphocytes to the tumor microenvironment." *PLoS One* **6**(5): e20267.
- Sariola, H. and M. Saarma (2003). "Novel functions and signalling pathways for GDNF." *J Cell Sci* **116**(Pt 19): 3855-62.
- Satake, K., Y. Matsuyama, et al. (2000). "Up-regulation of glial cell line-derived neurotrophic factor (GDNF) following traumatic spinal cord injury." *Neuroreport* **11**(17): 3877-81.

- Sawada, M., A. Suzumura, et al. (1990). "Activation and proliferation of the isolated microglia by colony stimulating factor-1 and possible involvement of protein kinase C." Brain Res **509**(1): 119-24.
- Schartner, J. M., A. R. Hagar, et al. (2005). "Impaired capacity for upregulation of MHC class II in tumor-associated microglia." Glia **51**(4): 279-85.
- Scheurer, M. E., M. L. Bondy, et al. (2008). "Detection of human cytomegalovirus in different histological types of gliomas." Acta Neuropathol **116**(1): 79-86.
- Schlachetzki, J. C., B. L. Fiebich, et al. (2010). "Norepinephrine enhances the LPS-induced expression of COX-2 and secretion of PGE2 in primary rat microglia." J Neuroinflammation **7**: 2.
- Schwartz, M., O. Butovsky, et al. (2006). "Microglial phenotype: is the commitment reversible?" Trends Neurosci **29**(2): 68-74.
- Sciame, G., A. Soriani, et al. (2010). "CX3CR1/CX3CL1 axis negatively controls glioma cell invasion and is modulated by transforming growth factor-beta1." Neuro Oncol **12**(7): 701-10.
- Shankaran, M., M. E. Marino, et al. (2007). "Measurement of brain microglial proliferation rates in vivo in response to neuroinflammatory stimuli: application to drug discovery." J Neurosci Res **85**(11): 2374-84.
- Sharif, A., P. Legendre, et al. (2007). "Transforming growth factor alpha promotes sequential conversion of mature astrocytes into neural progenitors and stem cells." Oncogene **26**(19): 2695-706.
- Shideman, C. R., S. Hu, et al. (2006). "CCL5 evokes calcium signals in microglia through a kinase-, phosphoinositide-, and nucleotide-dependent mechanism." J Neurosci Res **83**(8): 1471-84.
- Skuljec, J., H. Sun, et al. (2011). "CCL5 induces a pro-inflammatory profile in microglia in vitro." Cell Immunol **270**(2): 164-71.
- Sliwa, M., D. Markovic, et al. (2007). "The invasion promoting effect of microglia on glioblastoma cells is inhibited by cyclosporin A." Brain **130**(Pt 2): 476-89.
- Sofroniew, M. V. and H. V. Vinters (2009). "Astrocytes: biology and pathology." Acta Neuropathol **119**(1): 7-35.
- Soler, R. M., X. Dolcet, et al. (1999). "Receptors of the glial cell line-derived neurotrophic factor family of neurotrophic factors signal cell survival through the phosphatidylinositol 3-kinase pathway in spinal cord motoneurons." J Neurosci **19**(21): 9160-9.
- Song, H. and A. Moon (2006). "Glial cell-derived neurotrophic factor (GDNF) promotes low-grade Hs683 glioma cell migration through JNK, ERK-1/2 and p38 MAPK signaling pathways." Neurosci Res **56**(1): 29-38.
- Soroceanu, L., L. Matlaf, et al. (2011). "Human cytomegalovirus US28 found in glioblastoma promotes an invasive and angiogenic phenotype." Cancer Res **71**(21): 6643-53.
- Stallcup, W. B. and F. J. Huang (2008). "A role for the NG2 proteoglycan in glioma progression." Cell Adh Migr **2**(3): 192-201.
- Stiles, C. D. and D. H. Rowitch (2008). "Glioma stem cells: a midterm exam." Neuron **58**(6): 832-46.
- Tang, M. J., D. Worley, et al. (1998). "The RET-glial cell-derived neurotrophic factor (GDNF) pathway stimulates migration and chemoattraction of epithelial cells." J Cell Biol **142**(5): 1337-45.
- Teixeira, M. M., M. C. Vilela, et al. (2010). "Using intravital microscopy to study the role of chemokines during infection and inflammation in the central nervous system." J Neuroimmunol **224**(1-2): 62-5.

- Trebst, C., T. L. Sorensen, et al. (2001). "CCR1+/CCR5+ mononuclear phagocytes accumulate in the central nervous system of patients with multiple sclerosis." Am J Pathol **159**(5): 1701-10.
- Tremblay, M. E., B. Stevens, et al. (2011). "The role of microglia in the healthy brain." J Neurosci **31**(45): 16064-9.
- Trotter, J., K. Karram, et al. (2010). "NG2 cells: Properties, progeny and origin." Brain Res Rev **63**(1-2): 72-82.
- Trupp, M., C. Raynoschek, et al. (1998). "Multiple GPI-anchored receptors control GDNF-dependent and independent activation of the c-Ret receptor tyrosine kinase." Mol Cell Neurosci **11**(1-2): 47-63.
- Uludag, H., P. De Vos, et al. (2000). "Technology of mammalian cell encapsulation." Adv Drug Deliv Rev **42**(1-2): 29-64.
- Viola, A. and A. D. Luster (2008). "Chemokines and their receptors: drug targets in immunity and inflammation." Annu Rev Pharmacol Toxicol **48**: 171-97.
- Visted, T., R. Bjerkvig, et al. (2001). "Cell encapsulation technology as a therapeutic strategy for CNS malignancies." Neuro Oncol **3**(3): 201-10.
- Voisin, P., V. Bouchaud, et al. (2010). "Microglia in close vicinity of glioma cells: correlation between phenotype and metabolic alterations." Front Neuroenergetics **2**: 131.
- Walzlein, J. H., M. Synowitz, et al. (2008). "The antitumorigenic response of neural precursors depends on subventricular proliferation and age." Stem Cells **26**(11): 2945-54.
- Wan, G. and H. P. Too "A specific isoform of glial cell line-derived neurotrophic factor family receptor alpha 1 regulates RhoA expression and glioma cell migration." J Neurochem **115**(3): 759-70.
- Wan, G. and H. P. Too (2010). "A specific isoform of glial cell line-derived neurotrophic factor family receptor alpha 1 regulates RhoA expression and glioma cell migration." J Neurochem **115**(3): 759-70.
- Watters, J. J., J. M. Schartner, et al. (2005). "Microglia function in brain tumors." J Neurosci Res **81**(3): 447-55.
- Weischenfeldt, J. and B. Porse (2008). "Bone Marrow-Derived Macrophages (BMM): Isolation and Applications." CSH Protoc **2008**: pdb prot5080.
- Wells, S. A., Jr. and M. Santoro (2009). "Targeting the RET pathway in thyroid cancer." Clin Cancer Res **15**(23): 7119-23.
- Wen, P. Y. and S. Kesari (2008). "Malignant gliomas in adults." N Engl J Med **359**(5): 492-507.
- Wesolowska, A., A. Kwiatkowska, et al. (2008). "Microglia-derived TGF-beta as an important regulator of glioblastoma invasion--an inhibition of TGF-beta-dependent effects by shRNA against human TGF-beta type II receptor." Oncogene **27**(7): 918-30.
- Wick, W., M. Weller, et al. (2011). "Pathway inhibition: emerging molecular targets for treating glioblastoma." Neuro Oncol **13**(6): 566-79.
- Wiesenhofer, B., G. Stockhammer, et al. (2000). "Glial cell line-derived neurotrophic factor (GDNF) and its receptor (GFR-alpha 1) are strongly expressed in human gliomas." Acta Neuropathol **99**(2): 131-7.
- Wiesenhofer, B., C. Weis, et al. (2000). "Glial cell line-derived neurotrophic factor (GDNF) is a proliferation factor for rat C6 glioma cells: evidence from antisense experiments." Antisense Nucleic Acid Drug Dev **10**(5): 311-21.
- Wiggins, H. and J. Rappoport (2010). "An agarose spot assay for chemotactic invasion." Biotechniques **48**(2): 121-4.

- Wordinger, R. J., W. Lambert, et al. (2003). "Cells of the human optic nerve head express glial cell line-derived neurotrophic factor (GDNF) and the GDNF receptor complex." Mol Vis **9**: 249-56.
- Wu, A., J. Wei, et al. (2010). "Glioma cancer stem cells induce immunosuppressive macrophages/microglia." Neuro Oncol **12**(11): 1113-25.
- Wu, Y., Y. Lu, et al. (2012). "In silico experimentation of glioma microenvironment development and anti-tumor therapy." PLoS Comput Biol **8**(2): e1002355.
- Yasuhara, T., T. Shingo, et al. (2007). "Glial cell line-derived neurotrophic factor (GDNF) therapy for Parkinson's disease." Acta Med Okayama **61**(2): 51-6.
- Ye, X., Q. Zhao, et al. (2009). "Enhancement of mesenchymal stem cell attachment to decellularized porcine aortic valve scaffold by in vitro coating with antibody against CD90: a preliminary study on antibody-modified tissue-engineered heart valve." Tissue Eng Part A **15**(1): 1-11.
- Yoshida, H., T. Imaizumi, et al. (2007). "Retinoic acid-inducible gene-I mediates RANTES/CCL5 expression in U373MG human astrocytoma cells stimulated with double-stranded RNA." Neurosci Res **58**(2): 199-206.
- Zhai, H., F. L. Heppner, et al. (2011). "Microglia/macrophages promote glioma progression." Glia **59**(3): 472-85.
- Zhang, G. J., T. B. Chen, et al. (2008). "Bioluminescence imaging of hollow fibers in living animals: its application in monitoring molecular pathways." Nat Protoc **3**(5): 891-9.
- Zhang, J., S. Sarkar, et al. (2011). "A dialog between glioma and microglia that promotes tumor invasiveness through the CCL2/CCR2/interleukin-6 axis." Carcinogenesis **33**(2): 312-9.
- Zhang, W., W. T. Couldwell, et al. (1999). "Direct gap junction communication between malignant glioma cells and astrocytes." Cancer Res **59**(8): 1994-2003.
- Zhao, D., J. Najbauer, et al. (2008). "Neural stem cell tropism to glioma: critical role of tumor hypoxia." Mol Cancer Res **6**(12): 1819-29.
- Zhong, Y. and R. V. Bellamkonda (2008). "Biomaterials for the central nervous system." J R Soc Interface **5**(26): 957-75.
- Zhou, N., Z. Luo, et al. (2002). "Exploring the stereochemistry of CXCR4-peptide recognition and inhibiting HIV-1 entry with D-peptides derived from chemokines." J Biol Chem **277**(20): 17476-85.

## **9. Appendix**

### **Curriculum vitae**

Mein Lebenslauf wird aus Datenschutzgründen in der elektronischen Version meiner Arbeit nicht mit veröffentlicht.

## Publications

1. **Min-Chi Ku**, Susanne Wolf, Dorota Respondek, Vitali Matyash, Andreas Pohlmann, Sonia Waiczies, Helmar Waiczies, Thoralf Niendorf, Michael Synowitz, Rainer Glass, and Helmut Kettenmann. GDNF Mediates Glioblastoma Induced Microglia Attraction But Not Astrogliosis. In revision in *Acta Neuropathologica*.
2. Katyayni Vinnakota, Feng Hu, **Min-Chi Ku**, Pina Knauff, Andreas Pohlmann, Sonia Waiczies, Helmar Waiczies, Thoralf Niendorf, Nico van Rooijen, Seija Lehnardt, Uwe-Karsten Hanisch, Michael Synowitz, Darko Markovic, Susanne Wolf, Rainer Glass and Helmut Kettenmann. Toll-like-receptor 2 signaling mediates increase in MT1-MMP expression in glioma-associated microglia and contributes to glioma expansion. In preparation.
3. **Min-Chi Ku**, Chiao-Chi V. Chen, Antony Josphin Denslin Vinitha, Yi-Hua Hsu, Chien-Yuan Lin, Jiann-Shiun Lai, Taichang Jang, Lawrence D. Recht, and Chen Chang. Long-Term Characterization of Simple Magnetic Nanoparticle Labeled Neural Progenitor Cells. *BMC Cell Biology*. In revision in *PLOS ONE*.
4. Jia-Yi Wang, Li-Li Wen, Ya-Ni Huang, Yen-Tsun Chen and **Min-Chi Ku**. Dual Effects of Antioxidants in Neurodegeneration: Direct Neuroprotection against Oxidative Stress and Indirect Protection *via* Suppression of Glia-mediated Inflammation. *Current Pharmaceutical Design*. 12(27):3521-33. Review, 2006.
5. Yi-Wen Cheng, **Min-Chi Ku**, Chiu-Ming Ho, Chok-Yung Chai, and Chun-Kuei Su. GABA<sub>B</sub> receptor-mediated suppression of sympathetic outflow from the spinal cord of neonatal rats. *Journal of Applied Physiology*. 99(5):1658-67, 2005.
6. Jia-Perng Chen, Nan-Shih Liao, Szu-Liang Lai, Lilan Hsu, Wan-Yu Mao, **Min-Chi Ku** and Fang Liao. Reduced 2,4-dinitro-1-fluorobenzene-induced contact hypersensitivity response in IL-15 receptor  $\alpha$ -deficient mice correlates with diminished CCL5/RANTES and CXCL10/IP-10 expression. *European Journal of Immunology* 35(3): 690-698, 2005.

## Meetings and presentation

1. **M.C. Ku**, R. Glass and H. Kettenmann. Interaction of glioma cells with intrinsic brain cells  
(2009: MDC and FMP PhD retreat)
2. **M.C. Ku**, R. Glass and H. Kettenmann. Interaction of glioma cells with intrinsic brain cells  
(2010: MDC and FMP PhD retreat)
3. **M.C. Ku**, R. Glass and H. Kettenmann. Interaction of glioma cells with intrinsic brain cells  
(2009: Berlin Brain Days, Berlin, Germany)
4. **M.C. Ku**, R. Glass and H. Kettenmann. Interaction of glioma cells with intrinsic brain cells  
(2010: Berlin Brain Days, Berlin, Germany)
5. **M.C. Ku**, R. Glass and H. Kettenmann. Interaction of glioma cells with intrinsic brain cells  
(2011: 9<sup>th</sup> meeting of German Neuroscience Society, Göttingen, Germany)
6. **M.C. Ku**, R. Glass and H. Kettenmann. Interaction of glioma cells with intrinsic brain cells  
(2011: Brain Tumor meeting, Berlin, Germany)
7. **M.C. Ku**, R. Glass and H. Kettenmann. Interaction of glioma cells with intrinsic brain cells  
(2011: Berlin Brain Days, Berlin, Germany)
8. **M.C. Ku**, R. Glass and H. Kettenmann. Interaction of glioma cells with intrinsic brain cells  
(2011: Society of Neuroscience meeting, Washington DC, USA)
9. **M.C. Ku**, S. Wolf and H. Kettenmann. Interaction of glioma cells with intrinsic brain cells  
(2012: MDC evaluation, Berlin, Germany)

## Erklärung

„Ich, Min-Chi Ku, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „Interaction of glioma cells and intrinsic brain cells-soluble factor mediated“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Berlin, den

Min-Chi Ku