Glioma-Associated Mesenchymal Stem Cells Have Profound Effects on Brain Tumors

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For My Family

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II. ABREVIATIONS

AEG1	Astrocyte elevated gene 1
AML	Acute myeloid leukemia
ARF6	ADP-ribosylation factor 6
ARG1	Arginase-1
BM-MSC	Bone Marrow – Mesenchymal Stem Cell
BTSC	Brain Tumor Stem Cell
CD	Cluster of differentiation
CDKN2a	Cyclin Dependent Kinase Inhibitor 2A
cDNA	Complementary DNA
CM	Conditioned medium
CMV	Cytomegalovirus
CO_2	Carbon dioxide
Cre	Cre-recombinase
d	Days
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Di-methyl sulfoxide
E3	EFNA3 knockdown construct 3 and cells that received it
EFNA3	Ephrin receptor ligand A3
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor variant III
EMT	Epithelial to mesenchymal transition
ERK1/2	Early response kinase 1/2
ERT2	Mutated Estrogen receptor
ESCRT	Endosomal-sorting complex required for transport
Exo	Exosome
FCS	Fetal Calf Serum
Flip-GFP	Flipped form of GFP
GBM	Glioblastoma
GCV	Ganciclovir
GFP	Green Fluorescent Protein
GSC	Glioma stem cell
HO1	Heme oxygenase 1
HSC	Hematopoietic stem cell
IDH1	Isocitrate dehydrogenase 1
IL	Interleukin
ILV	Intraluminal vesicle
KD	Knockdown
KO	Knockout
LTR	Long terminal repeat
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid derived suppressor cell
miR	Micro RNA
ml	Milliliter
MMP	Matrix metalloprotease
MSC	Mesenchymal Stem Cell
MVB	Multivesicular bodies

ng	Nanogram
NO	Nitric oxide
NOS	Nitric oxide synthase
NPC	Neural precursor cell
NSC	Neural stem cell
NSF	N-ethylmaleimide sensitive fusion protein
NT	Non-targeting
OE	Overexpression
PA	Phospatidic acid
PDGFRA	Platelet-derived growth factor receptor alpha
PFA	Paraformaldehyde
PI3K	Phospatidyl-inositol 3 kinase
PTEN	Phosphatase and Tensin homolog
Puro	Puromycin
QKI	Quaking
RFP	Red fluorescent protein
RNP	Ribonucleoprotein
RTK	Receptor Tyrosine Kinase
SNARE	Soluble NSF-attachment protein receptor
St. dev.	Standard deviation
T25/T75/T150	Tissue culture flask with growth areas of 25, 75 and 150 cm^2
TC	Tissue culture
TK	Thymidine kinase
TMZ	Temozolomide
TrkA	Tyrosine kinase receptor type 1

1.INTRODUCTION

1.1. Glioblastomas

Glioblastomas (GBM) are devastating, treatment-refractory brain tumors [Westphal et al., 2011]. GBM can arise *de novo*, in patients with no prior clinical history for brain tumors or after malignant progression from more benign tumors. They are the most common malignant brain tumors comprising 16% of all primary central nervous system malignancies [Thakkar et al., 2012]. Although GBMs almost always occur in the brain, they can also appear in the brain stem, cerebellum, and spinal cord. 61 % of all GBMs are observed in the four lobes of brain: 25% frontal lobe, 20% temporal lobe, 13% parietal and 3% occipital lob [American Association of Neuroscience Nurses (AANN), 2014].

Initially all GBMs were thought to arise from glial cells, however, recent evidences indicate that a variety of cell types which have neural-progenitor-like properties might give rise to GBMs as well. Those cells could be at multiple stages of differentiation from neural stem cells to glial cells with different alterations in different signaling pathways [Parsons et al., 2008].

The genomic profiling that has been carried out from more than 200 human brain tumors has suggested more than 600 genes that have been altered. Those alterations converge onto three main signaling pathways that are commonly activated: p53 signaling pathway; receptor tyrosine kinase (RTK) / Ras / phosphoinositide-3 kinase (PI3K) signaling pathways; and retinoblastoma pathway [Chen et al., 2012]. The alterations in these pathways lead to constant activation of cell proliferation signals, and resistance to cell death signals (enhanced survival) while also enabling cells to escape from cell cycle checkpoints, senescence and apoptosis [Alifieris et al., 2015]. Additionally, molecular fingerprints have been identified between primary and secondary GBMs. Typical genetic alteration for primary GBMs are comprised of, but not limited to, epidermal growth factor receptor (EGFR) overexpression, phosphate and tensin homologue (PTEN) mutations, and loss of chromosome 10q. Secondary GBMs mostly harbor isocitrate dehydrogenase 1 (IDH1) mutations, p53 mutations, and chromosome 19q loss [Reya et al., 2001; Wilson et al., 2014; Young et al., 2015].

Based on the alterations in the signaling pathways, three subtypes of GBMs have been termed: Proneural, Classical and Mesenchymal. All three subtypes harbor EGFR amplification and CDKN2a deletion. Moreover, classical subtype carries p53 and EGFR mutations. In addition, EGFRvIII, an overactive EGFR variant, is frequently observed in classical subtype. In the proneural subtype, PDGFRA amplification dominates along with p53 and IDH1 mutations. IDHs function in krebs cycle of oxidative energy production pathway in cells. Mutations in IDH genes lower the activity of these enzymes causing higher exposure to free radicals leading to higher mutation rate in DNA of those cells, a common phenomenon in cancer cells [Pollard and Ratcliffe, 2009]. Mesenchymal subtype harbors p53 and NF1 mutations. In accordance with the subtype definitions, mesenchymal subtype has been shown to be the most aggressive of the three subtypes with a large infiltration potential and chemoresistance, followed by proneural and classical [Brennan et al., 2013].

Glioblastoma, being a solid tumor, harbors many different niches and different cells types within those niches, which altogether are termed the tumor microenvironment. The tumor microenvironment consists of brain tumor stem cells (BTSC) [Bao et al., 2006], endothelial cells and hypoxic regions [Fidoamore et al., 2016]; immune cells and immune modulatory cues [Ghosh et al., 2010]; astrocytes [Graeber et al., 2002]; neural stem/precursor cells (NPC) [Watters et al., 2005]; Mesenchymal Stem Cells (MSC) [Le et al., 2003; Aboody et al., 2000]. Each element within the tumor microenvironment provide the tumor cells with an intricate support enabling them to propagate and invade the surrounding tissue (**Figure 1**).

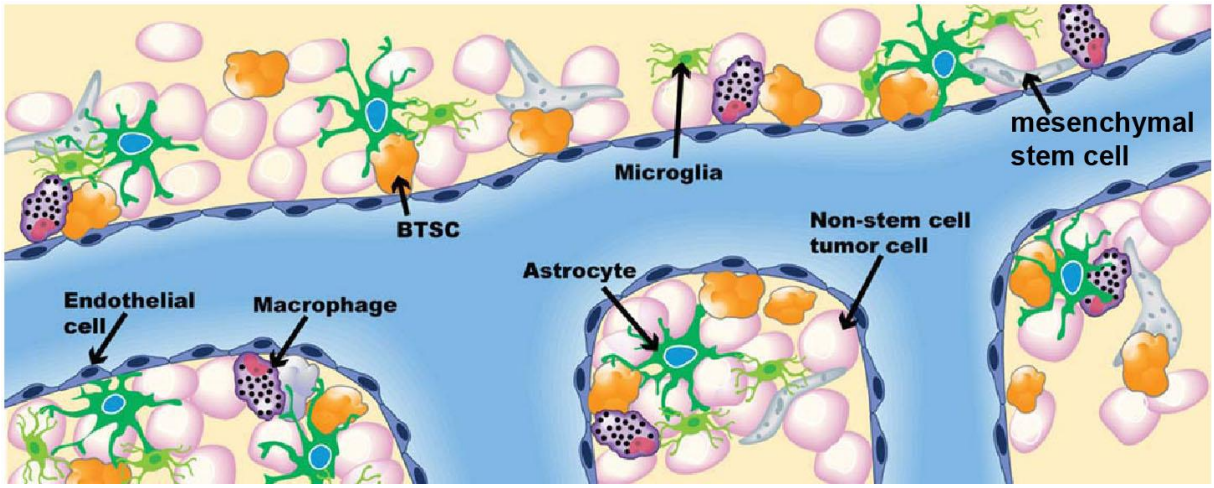


Figure 1: Many different cell types are present in the brain tumor microenvironment. Each cell type contributes to the tumor pathology in a unique way. Those cells include but not limited to brain tumor stem cells, endothelial cells, immune cells such as machrophages and microglia, astrocytes, neural stem / precursor cells and mesenchymal stem cells [Charles et al., 2011].

1.2. Glioma Microenvironment

1.2.1. Brain Tumor Stem Cells

Cancer stem cells were isolated initially from people with acute myeloid leukemia (AML) where they were able to reproduce many features of AML in immunodeficient mice [Lapidot et al, 1994]. Similar discovery was made in brain tumors by Ignatova et al, where they isolated clonogenic neurosphere-forming cells from human glioblastoma and medulloblastoma postsurgery specimens and demonstrated the presence of cells with stem-like properties in brain tumors which later was termed Brain Tumor Stem Cells or Glioma Stem Cells (BTSCs or GSCs) [Ignatova et al, 2002]. This finding was further supported by Singh et al, where they proved the existence of a $CD133^{+}$ cell population capable of forming tumors *in vivo* and neurosphere formation *in vitro* [Singh et al, 2004].

GSCs are capable of self-renewal, multiple drug resistance and radiation resistance and high tumorigenicity along with expression of many stem cells markers. Such as c-Myc, SOX2, OCT4, NANOG, SALL4, STAT3, Bmi1, and KLF4 [Wang et al, 2008]. It has also been demonstrated that only four transcription factors such as POU3F2, SOX2, SALL2, and OLIG2 are enough to induce complete transformation of differentiated GBMs into GSCs [Suvà et al, 2014]. In addition to stem cell markers, the implicated signaling pathways in GSCs, which are activated frequently via dysregulations or mutations, mediating the self-renewal and the multipotency are Notch, sonic hedgehog (SHH) and Wnt/b-catenin pathways and those pathways are shared with the NSCs [Yi et al, 2016]. Moreover, Quakings (QKI) and Eph-Ephrin receptor ligand systems have emerged as part of the key signaling pathways in GSCs with potential to promote brain tumors due to dysfunctions.

The QKIs belong to the heteronuclear ribonucleoprotein particle K (hnRNPK) homology (KH) domain family of RNA binding proteins [Bockbrader and Feng, 2008]. The QKI locus encodes for three major alternatively spliced genes that share RNA-binding KH domain and differ in their C-terminal 30 aminoacids, namely QKI-5, QKI-6 and QKI-7 [Ebersole et al., 1996]. QKI-5 carries a nuclear localization signal targeting this isoform predominantly to nucleus whereas QKI-6 and QKI-7 are localized in the cytosol [Pilotte et al., 2001; Wu et al., 2002]. QKIs selectively interact with what are called quaking response elements (QRE) localized to intronic regions and mRNAs, playing roles in a variety of steps of RNA processing machinery controlling stability, translation and localization. They are pivotal during developmental decision processes and impact the glial, oligodendrocyte, and Schwann cell differentiation and myelination of the nervous system [Hafner et al., 2010; Galerneau and Richard, 2005; Chenard and Richard, 2008; Bockbrader and Feng, 2008].

QKIs have been shown to suppress tumorigenicity in various cancers such as colorectal cancer [Ji et al., 2013], clear cell renal cell carcinoma [Zhang et al., 2016] and lung cancer [Zhou et al., 2017] via suppression of several signaling pathways involved in proliferation and invasion. In brain tumors, a dichotomous role has been attributed to QKIs. They have been shown to inhibit GBM tumorigenesis by Li et al., Chen et al., and Shingu et al. [Li et al., 2002; Chen et al., 2012; Shingu et al., 2017]. On the other hand, Bandopadhayay et al. and Wang et al. demonstrated QKIs to promote gliomagenesis by enhancing proliferation [Wang et al., 2013; Bandopadhayay et al., 2016]. Therefore, it is still a controversial issue to define QKIs as pro- or anti-tumorigenic genes in GBM biology.

Eph receptors constitute the largest group of receptor tyrosine kinases (RTK). They are transmembrane proteins which transduce signals from cell exterior to cytosol via the activation of their kinase domain within the cytosol upon binding of their ligands, Ephrins, to the extracellular domains. Ephrins are also membrane bound ligands, therefore, Eph-Ephrin signaling mediates cell-cell contact-dependent communication. They confer a bidirectional signaling affecting both the stimulant and the stimulated cell [Lisabeth et al., 2013].

Eph receptors and Ephrins are expressed literally in all the tissue types and are involved in a variety of processes, especially in developmental mechanisms governing cardiovascular and skeletal development, axon guidance and tissue patterning [Palmer and Klein 2003]. Eph/Ephrin signaling converges mostly on cell adhesion, cell sorting during embryogenesis,

growth cone retraction in axon guidance and cell migration [Arvanitis and Davy, 2018]. Recently they have also been implicated in learning and memory [Gerlai, 2002], insulin secretion [Konstantinova et al., 2007] and bone homeostasis [Zhao et al., 2006]. Alterations in Eph/Ephrin system often leads to cancer [Pasquale 2005].

Eph receptors contain typical RTK structure with an extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic region with the kinase domain (Figure 2). There are nine EphA receptors in human genome interacting redundantly with five EphrinA ligands; and five EphB receptors partnering with three EphrinB ligands [Pasquale 2004; Paquale 2005]. Additionally, some Eph receptors have alternatively spliced forms with distinct roles [Zisch and Pasquale 1997]. Both EphrinAs and EphrinBs are composed of a conserved Eph receptor binding domain. EphrinAs are linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor, and they have been shown to be released from the plasma membrane to activate their EphA receptor partners at distant sites [Bartley et al. 1994; Wykosky et al. 2008]. Different from EphrinAs, EphrinBs contain a transmembrane region followed by a short cytosolic part [Holen et al., 2011].

Upon binding of Ephrins, Eph receptors oligomerize by interactions at various domains and start a signaling cascade within the cytosol, which is called the forward signaling [Barton et al., 2013; Himanen et al., 2010]. This clustering involves interactions with actin cytoskeleton and may include Eph receptors of both A and B types [Janes et al., 2011]. Oligomerization leads to trans-phosphorylation of two conserved tyrosine residues in the juxtamembrane domain relieving the inhibitory intramolecular interactions via a conformational change and activating the kinase activity (Figure 3) [Binns et al., 2000; Zisch et al., 2000]. Eph receptors modulate many of the same effectors which function downstream of the other RTK families, however, Eph receptor signaling differs from RTK signaling due to their oligomerization mechanism [Wagner et al., 2013]. Activation of Eph receptors leads to recruitment of downstream signaling proteins that contain SH2 domains such as nonreceptor tyrosine kinases of the Abl and Src family as well as adaptors like Nck and Crk [Pasquale, 2010]. Moreover, PDZ domain carrying Rho and Ras GTPases and Akt/mTORC1 effectors bind to carboxy terminals of Eph receptors. Most RTKs utilize those signaling molecules to induce cell proliferation, survival and forward movement whereas Eph receptors cause inhibition of cell growth and activate cell repulsion [Lisabeth et al., 2013].

In addition to forward signaling commenced by the Eph receptors, Ephrin ligands also lead to a signaling cascade in the cells they are bound to, which is termed reverse signaling [Pasquale, 2010]. In reverse signaling, Ephrin-B ligands are phosphorylated by Src kinases creating binding sites for adaptors such as Grb4. Ephrin-B signaling via Gbr4 modulates axone-pruning, synapse formation and dentritic spine morphogenesis in developing hippocampus [Cowan and Henkmeyer, 2001; Xu and Henkmeyer, 2009]. Binding of PDZ-domain-containing adaptors such as PDZ-RGS3 to carboxy terminal tail of Ephrin-B enables it to utilize G-protein coupled receptors that control neural cell migration and neural progenitor cell self-renewal [Lu et al., 2001; Qiu et al., 2010]. The Ephrin-As do not possess a cytoplasmic domain which can interact with intracellular signaling proteins which renders the signaling via Ephrin-As puzzling. However, studies in neurons have demonstrated that they interact with neurotropin receptor

p75 and Ret-RTKs and the TrkBs as transmembrane interaction partners thereby leading to axonal guidance and branching [Lim et al., 2008; Bonanomi et al., 2012]. Consequently, via those interactions and other possible mechanisms, Ephrin-As are involved in many physiological processes such as inhibition of neural progenitor cell proliferation by Ephrin-A2 [Holmberg et al., 2005]; modulation of glutamate uptake in glial cells in hippocampus thereby controlling synaptic plasticity by Ephrin-A3 [Filosa et al., 2009]; inhibition of apoptotic cell death in Jurkat immune cells via activation of Src family kinases and Akt by Ephrin-A4 [Holen et al., 2008] and control of insuling signaling in pancreatic β cells by Ephrin-A5 [Konstantinova et al., 2007]. Ephrin-A5 also increases cell-substrate adhesion in fibroblasts via activation of Src family kinase Fyn and integrins thereby controlling invasiveness which might be important in cancer cells as well [Campbell et al., 2006].

Identification of Eph/Ephrin system and cancer dates back to when the first Eph family member, EphA1, was isolated from a carcinoma cell line [Hirai et al., 1987]. EphrinA1 ligand was identified in the same way a few years later [Bartley et al., 1994]. The link between Eph/Ephrin system and cancer has largely accumulated over the past years. Multiple Eph receptors and Ephrin ligands are expressed within the tumor and tumor microenvironment. They have large impacts on tumor behavior via promotion of aberrant cell-cell as well as tumor-microenvironment communication [Surawska et al., 2004; Ireton et al., 2005]. Various Eph/Ephrin types have been reported in many cancer types such as prostate cancer [Huusko et al., 2004], colorectal cancer [Zagopoulos et al., 2008], cervical cancer [Narayan et al., 2003] and lung cancer [Frohling and Dohner, 2008]. Eph/Ephrin system is frequently compromised in brain tumors. Moreover, expression of some Eph/Ephrin pairs have been shown to be altered in some gliomas and due to those alterations they were suggested as possible markers for gliomas [Wykosky et al., 2005; Wang et al., 2008]. EphA2 is overexpressed in GBMs although it is not detected in normal brain regions and it has been correlated with poor prognosis as it promotes tumor cell proliferation and tumor angiogenesis [Wykosky et al., 2005; Liu et al., 2006]. Additionally, EphB2 overexpression in U-251 MG cells stimulated migration and invasion [Wang et al., 2012]. EphA3 has also been shown to be elevated in 40% of GBM tissues, especially in mesenchymal subtype, and suggested to maintain stem cell properties of GSCs and tumorigenicity. Furthermore, EphrinB2 has been suggested to be a tumor promoter due to poor survival of patients with high EphrinB2 expression [Nakada et al., 2010]. Additionally, EphrinA1 stimulation of EphA2 overexpressing GBMs increased aggressiveness by enhancing invasive behaviors [Cheng et al., 2002]. In addition to basic research for uncovering the roles of Eph/Ephrin system in GBMs, some intervention studies have been carried out. For instance, chIIIA4 α-EPHA3 mAb [Day et al., 2013] was used to target specifically tumor stroma and inhibited tumor growth by disrupting stromal architecture [Vail et al., 2014]. Moreover, EphrinA1-Fc treatment depleted the tumor propagating cell population, inhibited self-renewal and induced astroglial differentiation of GBMs [Binda et al., 2012]. Most of those studies, however, were focused on the intratumoral expression of Eph receptors and Ephrins and are limited mostly to Eph receptors and few Ephrin ligands. We still have a lot to uncover about the mechanisms and ways of exploitations of Eph/Ephrin systems by cancer cells and especially in GBMs.

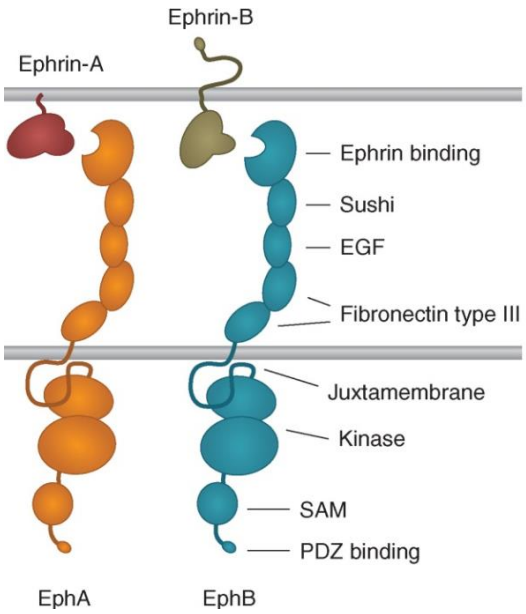


Figure 2: Domains and structure of Eph receptors and Ephrin Ligands. EphAs EphBs and EphrinBs contain transmembrane domains. EphA and EphB contains kinase domains as well as some adaptor domains where they interach with adaptors of RTK signaling pathway [Lisabeth et al., 2013].

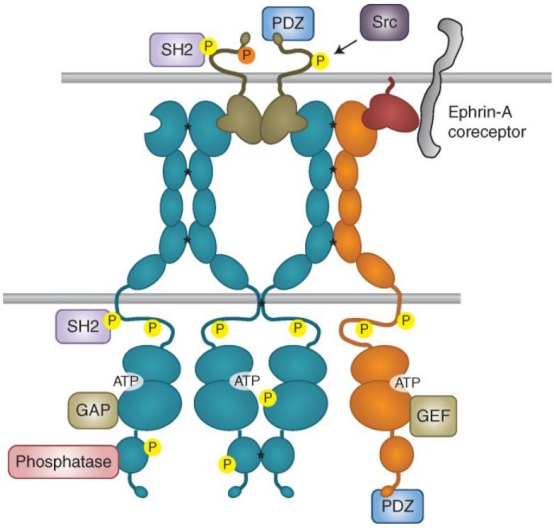


Figure 3: Bidirectional Clustering of Eph receptors and Ephrin Ligands. Upon contact, both EPh receptors and Ephrin Ligands interact at cis interaction domains (Shown by asterisks). Tyrosine phosphorylations are shown with yellow circles, Serine phosphorylations depicted by orange circles. Interation partners are also shown [Lisabeth et al., 2013].

1.2.2. Endothelial Cells and Hypoxic Regions

Although it is a common phenomenon observed in all solid tumors, glioblastomas are the most vascularized ones. This situation is brought about by the vascular proliferation known as microvascular hyperplasia which is a hallmark of GBM [Brat et al, 2003]. In microvascular hyperplasia, endothelials cells rapidly proliferate forming microaggregates of small blood vessels and smooth muscle cells / pericytes called glomeruloid bodies [Brat et al, 2003]. These structures differ from the normal blood vessels due to irregular structuring of blood vessels within GBM tissue, characterized by dilatations, incomplete or absent basement membranes, high permeability, irregular architecture, blind ends, absence of vascular smooth muscle and pharmacological / physiological receptors [Vaupel et al, 1989; Vaupel et al, 2004].

Caused by the irregularities in the vessel structure and the incomplete (open-end blood vesssels) circulation, the oxygen is not delivered homogeneously within the tumor where tumor cells spread rapidly going past the diffusion distance of the O_2 and nutrients. This

situation induces secretion of hypoxia inducible factors which in turn induces angiogenesis leading to formation of new blood vessels. However, the newly-formed capillaries will not provide enough blood flow for proper distribution of O_2 leading to an O_2 gradient within the neoplasm causing hypoxic or anoxic regions in tumor parenchyma [Vaupel et al, 2004]. The severe hypoxic areas frequently delineate the necrotic regions which are another hallmark of the solid tumors [Jensen et al, 2009; Heddleston et al, 2010]. The tumor necrosis takes place due to increased apoptosis or uncontrolled growth beyond the reach of blood supply [Brat et al, 2003]. Interestingly, the tumor size does not play any roles in the degree of intratumoral necrosis as it is present in both small and large solid tumors [Jensen et al, 2009].

Tumor cells under hypoxia display either a decreased proliferation accompanied by possible activation of apoptotic pathways or adaption to the stress which makes them more aggressive [Vaupel et al, 2008]. This adaptation towards aggressiveness is mediated by drastic changes in the gene expression profile which governs cellular events such as proliferation, glycolysis, angiogenesis, metastasis and invasion [Vaupel et al, 2004; Lu et al, 2010; Semenza et al, 2012]. In addition to becoming more metastatic and invasive with the alteration of gene expression profile upon hypoxia, tumor cells also become more resistant to conventional treatments such as chemotherapy and radiotherapy [Semenza et al, 2012]. The resistance to chemotherapy is thought to be caused by the decreased potential of cellular proliferation (whereby reducing the effectiveness of some chemotherapeutic agents that require cellular proliferation to take effect), the reduced activity of chemotherapeutic agents in hypoxic conditions, tissue acidosis and/or dampened apoptotic potential of cells [Hockel et al, 2001]. The hypoxia-induced radioresistance is proposed to be multi-factorial with decrease of partial oxygen pressure being the most likely mechanism followed by the higher levels of heat-shock proteins and decrease of apoptotic potential of cells.

GSCs are located close to the vascular niche of the tumor mass where they intimately interact with the endothelial cells and the blood which is reminiscent of normal stem cell behavior [Tavazoie et al, 2008]. In fact, the population size of GSCs which are in close contact with the blood vessels increases with the grade of the brain tumor which also applies to all GBM cells [Calabrese et al, 2007]. Furthermore, GSCs have been shown to travel along the blood vessels and transiently pause at vascular branch points for proliferating [Farin et al, 2006]. To further support the interaction between GSCs and the endothelial cells, Calabrese et al demonstrated the signaling from endothelial cells to GSCs which increased the stem cell – like properties of GSCs along with their tumorigenicity [Calabrese et al, 2007]. Nitric oxide (NO) is one of those factors capable of enhancing the self-renewal of GSCs in the perivascular niche. NO activates Notch signaling in GSCs whereby increasing stem-cell properties of those cells *in vitro* and tumor formation capacities *in vivo* [Charles et al, 2010]. Moreover, $CD133^+$ BTSCs secrete Vascular Endothelial Growth Factor (VEGF) which promotes the formation of new blood vessel tubular structure *in vitro* increasing their tumorigenicity *in vivo*. In return, endothelial cells secrete cues that increase the expression of stem cell markers in GSCs such as Olig2, Bmi1, Sox2 and CD133 [Yan et al, 2014]. In the same direction, upon the treatment of mice, which have received $CD133^+$ GSCs, with VEGF signaling inhibitor bevacizumab, the tumor growth and vascularization slowed down significantly [Bao et al, 2006]. This coupled system forms a signaling loop which favors the stem cell – like properties of GBMs and the tumor growth.

1.2.3. Immune Cells and Immune Modulatory Cues

GBMs secrete many cytokines, chemokines and growth factors, which eventually attract many other cell types from the vicinity as well as from circulation and promote their infiltration. Those cell types include a range of immune cells such as microglia, peripheral macrophages, myeloid-derived suppressor cells (MDSC), and regulatory T cells (Tregs) [Fecci et al, 2006; Lohr et al, 2011; Alexiou et al, 2013; Wainwright et al, 2013]. Those cells are reprogrammed with locally secreted cues thereby acquire new behaviors which either are inflammatory or anti-inflammatory. Moreover, GBMs, just like other solid tumors, are capable of inhibiting the host anti-tumor responses in multiple ways.

The resident macrophages of the brain are termed microglia. Those cells localize to brain during early development and form the ramified microglia thereafter [Hanisch and Kettenmann, 2007]. The activity of microglia depends on the type of pathology. In glioma microenvironment, microglia are thought to be inactive due to the immunosuppressive cytokines secreted by GBM, such as IL-10, IL-6, IL-4, TGF-β and Prostaglandin E2 [Wei et al, 2010]. Additionally, microglia express low levels of MHC Class II molecule along with some other costimulatory molecules [Badie et al, 2002]. When stimulated with lipopolysaccharides (LPS) and IFN-γ microglia assume M1 phenotype to secrete pro-inflammatory cytokines such as TNF-α, IL-1β and IL-12, present antigen and express high levels of inducible NO (iNOS) for NO production. This phenomenon takes place to kill pathogens and induce T cells for adaptive immune response [Gordon and Taylor, 2005]. In addition to M1 phenotype, microglia (and macrophages) display an M2 phenotype where they express anti-inflammatory cytokines such as IL-4, IL-10, IL3 and TGF-β, as well as Arginase-1 (Arg1) and CD206 which then leads to allergy response, parasite clearance, inflammatory dampening, tissue remodeling, angiogenesis, immune regulation and tumor promotion [Villalta et al., 2009]. In this respect, microglial cells seem to acquire M2 phenotype with the increasing histological malignancy. M2 phenotype represents the homeostatic state while M1 phenotype is a sign of inflammation. Given these facts, in GBM microenvironment, microglia assume an activated morphology but rather a different phenotype from that of a regular inflammation [Komohara et al, 2008; Charles et al, 2011]. Yet, microglia mediate tumor cell migration and tumor growth via MT1-MMP secretion in response to cues released from glioma cells, a phenomenon observed only when microglia are in the glioma-induced state [Markovic et al, 2005; Sliwa et al, 2007]. However, there are two contradictory studies where depletion of microglia, by Markovic et al, resulted in 80% decrease in tumor volume, while macrophage depletion, by Gallernau et al, induced 33% increase in the tumor volume, showing that myeloid depletion is both pro- and anti-tumorigenic due to potential unaccounted targeting of additional factors [Markovic et al, 2009; Galarneau et al, 2007].

In addition, Tregs infiltrate the GBM and suppress the immune responses in the GBM via heme oxygenase – 1 [El Andaloussi and Lesniak, 2007]. MDSCs inhibit antigen-specific $CD8^+$ T-Cells via generation of reactive oxygen species; inhibit T-cell proliferation and promote T-Cell apoptosis via nitric oxide synthase (NOS) and arginase-1 (ARG1) generating reactive nitrogen species [Marvel et al, 2015].

1.2.4 Astrocytes

Astrocytes are known for their roles in the maintenance of brain homeostasis as well as the blood-brain-barrier where they ensheath the endothelial cells with their end-feet (Kim et al, 2006). Additionally, they have been implicated in the development of brain tumors where reactive astrocytes interact with the brain tumors [Le et al, 2003]. Astrocytes secrete some neurotrophic factors such as TGF-α, CXCL12, S1P and GDNF which support the brain tumor cell growth [Hoelzinger et al, 2007]. Moreover, cytokines such as TGF-β and IL-6 secreted from astrocytes have been shown to promote the tumor cell proliferation in brain, *in vitro* [Sierra et al, 1997].

Tumor-associated astrocytes activate a metalloproteinase called proMMP2 aiding the glioma cells with their invasion [Le et al, 2003]. SDF1/CXCR-4 signaling is postulated to be an important factor in glioma cell proliferation as this signaling pathway is frequently over-activated in gliomas [Barbero et al, 2002]. Because the astrocytes also secrete SDF-1 [Bajetto et al, 1999] they are thought to be involved in the promotion of tumor development.

Astrocyte elevated gene 1 (AEG-1), which has been demonstrated to be elevated in adult astrocytes [Kang et al, 2005], has been implicated in the metastatic progression of brain tumors. AEG-1 was found to be frequently overexpressed in brain tumors [Emdad et al., 2007] and suppression of AEG-1 hindered the brain tumor growth in mice [Emdad et al., 2010]. Additionally, it was shown that, AEG-1 activity requires MMP2 and MMP9 [Emdad et al., 2010; Liu et al., 2010].

1.2.5. Neural Stem/Precursor Cells

The neural stem and precursor cells (NPCs) are resident stem cells of the brain which reside in subventrical zone and the subgranular zone of the dentate gyrus [Altman and Das, 1967; Reynolds and Weiss, 1992; Gage, 2000]. These cells are able to generate many cell types of the central nervous system via asymmetric division and differentiation throughout the development of the CNS [Reynolds and Weiss, 1992; Cameron et al, 1993]. Additionally, neurogenesis in the dentate gyrus is thought to be important for the memory formation and functions in the adulthood [Bruel-Jungerman et al., 2007].

In the last decade, NPCs have been postulated to be the origin of GBMs. However, the GBMs emerge in human brains long after the stem cell activity of NPCs cease. It has been shown by many research groups that NPCs migrate towards primary brain tumors and secrete anti-tumorigenic substances which are beneficial for the overall survival of the patients [Assanah et al., 2006; Assanah et al., 2009; Walzlein et al., 2008; Glass et al., 2005]. Stock et al. showed in 2012 that, the factors which suppress the brain tumors are endovanilloids [Stock et al., 2012]. Endovanilloids act on a non-selective cation channel called Transient Receptor Potential Vanilloid - 1 (TRPV1) [Stock et al., 2012]. Overall, NPCs in the brain tumor microenvironment are beneficial for the treatment and survival.

1.3. Mesenchymal Stem Cells in Tumor Microenvironment

1.3.1. Mesenchymal Stem Cells

Friedenstein was the first person to describe the multipotential stromal cells in the bone marrow where he described the isolation of spindle-shaped, clonogenic cells which he defined as colony forming unit fibroblasts (CFU-F). He demonstrated that those cells can be used as feeder cells for hematopoietic stem cells (HSC) and *in vivo* they can differentiate into adipocytes, osteocytes and chondrocytes [Friedshein et al, 1976]. Later on, Caplan termed those cells as mesenchymal stem cells based on their self-renewal and multilineage differentiation capacities [Caplan et al, 1991]. Further research into the biology of those cells has shown that, those cells are not only predecessors of cells from mesenchymal lineage, but also can transdifferentiate into other embryonic lineages (ectoderm and endoderm) [Pittenger et al, 1999]. Although the bone marrow is the place where most MSCs reside, research showed that, MSCs are found literally in all postnatal tissues adipose and chondroitin being the most well-known tissues [da Silva et al., 2006].

1.3.2. Defining MSCs

MSCs can be identified by some visible criteria of proliferation in the *in vitro* cell culture as an adherent population, fibroblast-like morphology (Figure 4), being able to form colonies *in vitro*, and ability to differentiate into three major lineages: osteocytes, chondrocytes and adipocytes [Horwitz et al, 2005; Caplan et al, 2011]. In addition to these superficial properties, it is possible to define MSC with presence and absence of a series of certain markers as they do not possess one unique marker. The consensus is that the human MSCs lack hematopoietic markers such as CD45, CD34, CD14 or co-stimulatory ones such as CD80, CD86 and CD40 while displaying variable levels of CD105 (endoglin), CD44, CD71 (transferrin receptor), CD73 (ecto-5'-nucleotidase), CD90 (THY1), CD271 (low-affinity nerve growth factor receptor), and the ganglioside GD2 and STRO-1 (anti-STRO1 monoclonal antibody for FACS) [Dominici et al, 2006]. The variations in the expression levels could be due to species differences, tissue sources and culture conditions.

Upon transplantation, MSCs in the bone marrow, can differentiate into pericytes, myofibroblasts, bone-marrow stromal cells, osteocytes, osteoblasts and endothelial cells, which all together contribute to the formation of a HSC niche [Muguruma et al, 2006]. The MSCs in the HSC niches keep the developing hematopoietic cells in a quiescent stage (at G0 phase) until a need arises and the signal arrives for them to terminally differentiate and to be released to the vascular system [Wilson and Trump, 2006]. In addition, by differentiating into osteoblasts and endothelial cells, MSCs shield HCSs from differentiation and/or apoptosis signals thereby promoting HSC stemness [Sacchetti et al, 2007].

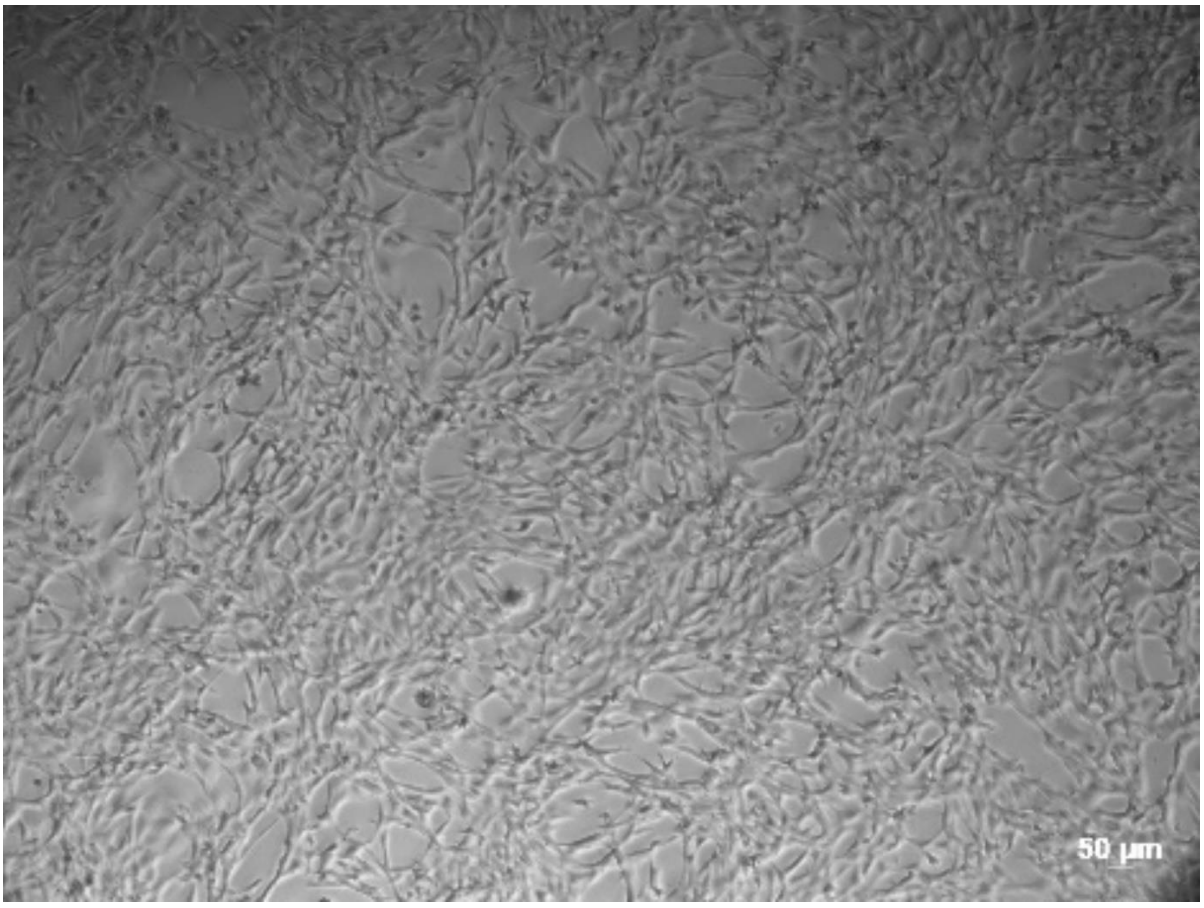


Figure 4: MSCs display fibroblast like morphology with adherent and elongated cell structure

1.3.3. Roles of MSCs in Homeostasis

It is well-known that MSCs have profound effects on the immune system and wound healing processes [Caplan and Correa, 2011; Bernardo and Fibbe, 2013; Otero-Vinas, 2016]. MSCs inhibit T-cell proliferation [Di Nicola et al., 2012], maturation of hematopoietic progenitors and monocytes into dendritic cells [Jiang et al., 2005] as well as DC activity [Aggarval et al, 2005], suppress cytotoxic activity of Natural Killer (NK) cells and interferon (IFN) release [Spaggiari et al, 2006]. MSCs coordinate two important phases of wound healing. In the first step, the blood-borne molecules leak from blood vessels to the surrounding whereby activating MSCs which in turn “turns on” an inflammatory response to clear out potential infections. In the second phase, after the infection risk is overcome, MSCs activate the local stem cells to promote the scar formation and the tissue healing [Caplan and Correa, 2011; Bernardo and Fibbe, 2013; Otero-Vinas, 2016].

1.3.4. Tumor-associated MSCs

The similarity between a tumor and a wound is well established when the damage to the surrounding and bleedings are taken into account [Dvorak et al., 1986]. It would be a reasonable conclusion if MSCs were assumed to home tumors, just as they home injury sites. Wallace et al in 2001, demonstrated the existence of MSCs in the primary tumor site of

multiple myeloma [Wallace et al., 2001]. They were located in the perivascular niche of the tumor and were morphologically and genetically distinct from tumor cells [Wallace et al., 2001]. The existence of MSCs was later demonstrated by more research groups in different tumors, such as lung, breast and prostate cancers by Gottschling et al, Lamb et al, and Santamaria-Martinez et al, respectively [Gottschling et al., 2013; Lamb et al., 2005; Santamaria-Martinez et al., 2009]. However, the mechanism by which MSCs migrate and settle in tumors has long been a mystery, until when MSCs were shown to migrate upon signaling via cytokine/receptor pairs such as SDF-1/CXCR4, SCF-c-Kit, HGF/c-Met, VEGF/VEGFR, PDGF/PDGFr, MCP-1/CCR2, and HMGB1/RAGE [Ries et al., 2007; Karp et al., 2009; Baek et al., 2011; Momin et al., 2010]. Moreover, when the amount of chemokine response elements on the surface of MSCs was increased, MSCs displayed an enhanced migration towards tumors upon systemic infusion [Shi et al., 2007]. Moreover, in 2009, Kidd et al first demonstrated the migration of luciferase-tagged MSCs to breast cancer xenografts upon systemic infusion [Kidd et al., 2009]. These phenomena have led to a deeper investigation of MSCs being used as anti-cancer agent delivery tools.

In the brain, the presence of MSCs was proven in 2012 by Paul et al, where they characterized the adult brain perivascular mesenchymal cells, demonstrating the lack of neural stem cell, hematopoietic, endothelial, neuronal and glial markers. Moreover, these cells were able to differentiate into cells of mesodermal lineage [Paul et al., 2012]. In terms of brain tumors, however, the presence of MSCs has been demonstrated by monitoring the migration of MSCs into brain tumors in experimental situations [Birnbaum et al., 2007; Hata et al., 2010; Shinojima et al., 2013]. In line with this and the other tumor types, upon flow cytometry analyses, GBM biopsies have been shown to contain a vast amount of MSCs, which also brings about one of the pathological hallmarks of GBM [Kim et al., 2013; Behnan et al., 2014]. Hossain et al. further investigated those MSCs isolated from patient biopsies [Hossain et al., 2016].

1.3.5. Role of MSCs in Tumor Niche

It has been a controversial issue what the role of MSCs in tumor niche is. It was first speculated that MSCs invade the tumor sites as there exist incomplete vessels and blood-brain-barrier is not intact so the term wounds that do not heal have been introduced for tumors [Dvorak et al., 1986] and this situation causes an inflammatory response due to leakage of tumor associated molecules into the bloodstream. Additionally, this leakage enables metastatis to other parts of the body. Moreover, MSCs take part in the maintenance and growth of the perivascular niche. However, soon after the MSCs had been introduced as part of the tumor microenvironment, research lead to different findings. It has been demonstrated that, BM-MSCs promote tumorigenesis of breast cancer stem cells. Throughout their research, Kornaub et al found that BM-MSCs increased the migration and invasion capacities of the breast cancer stem cells which was brought about by the Chemokine Cytokine Ligand 5 (CCL5) [Kornaub et al., 2007]. Additionally, similar effects of MSCs were shown in colon cancer where MSCs resulted in an elevated invasive behavior via IL-6 and in prostate cancer where MSCs contributed to progression and metastasis via TGF-β [Shinagawa et al., 2010; Lin et al., 2013; Ye et al., 2012; Roodhard et al., 2011]. MSCs also caused increased immunosuppression by the tumors which eventually lead to increased tumor burden [Djouad et al., 2003].

Furthermore, MSCs resulted in an elevated chemoresistance in breast cancer [Roodhard et al., 2011].

In gliomas, MSCs display profound effects. Glioma stromal mesenchymal stem cells promoted increased microvasculature in orthotopic xenografts of GBM thereby increasing angiogenesis and aggressiveness [Kong et al., 2013]. Moreover, brain tumor-derived MSCs increase the growth of GBMs [Behnan et al., 2014]. Breznik et al showed that BM-MSCs caused an increased invasive phenotype of GBM xenografts [Breznik et al., 2017]. Additionally, Hossain et al demonstrated that Glioma-Associated MSCs act on GSCs via soluble factors and promote tumor growth and aggressiveness [Hossain et al., 2016]. In contrast, MSCs have anti-tumorigenic effects on GBMs. BM-MSCs mediate anti-angiogenic effects via downregulation of PDGF/PDGFR signaling axis [Ho et al., 2013]. Cord blood MSCs upregulate the PTEN in GBM cells, leading to a downregulation of PI3K/Akt signaling which in turn inhibits the migration of GBMs [Dasari et al., 2010]. Furthermore, amniotic membrane – derived MSCs greatly increased the expression of Bax, Caspase 8 and Caspase 3 whereas decreased Bcl2 expression thereby inducing the GBM cell apoptosis [Jiao et al., 2012].

Consequently, two conflicting roles of MSCs in GBMs proposed by the GBM community: (1) MSCs are a possible target for adjuvans treatments of GBM; and (2) MSCs should be exploited (to deliver therapeutic means) to cure GBMs. Because of these discrepancies, MSC-related therapies are difficult to establish [Mendicino et al., 2014]. In order to overcome these problems, it is imperative for MSC research to focus more on the reasons of these variations and to shed light on the circumstances under which MSCs are pro- or anti-tumorigenic as well as the further signaling pathways which generate those afore-mentioned dichotomous roles. Moreover, an enormous proportion of the research about GBM – MSC interaction is limited to characterization of soluble factors secreted from MSCs and act on GBMs. When considering such paracrine interactions of MSCs, exosomal communication is often overlooked in terms of the roles of non-tumor cell exosomes on GBMs [Figueroa et al., 2017]. Therefore, the knowledge to be gained from exosomes of MSCs might prove useful in revealing the mechanisms of interactions between MSCs and GBMs which in turn might be pivotal for understanding the dichotomous roles of MSCs in GBM parenchyma as there would be more factors to consider in terms of glioma physiology and pathogenesis.

1.4. Exosomes

Cell-to-cell communication has previously been known to be limited to transfer of ions, lipids, hormones and proteins from one cell to another by means of extracellular release or via gap junctions [Von Euler et al., 1936; Cohen et al., 1954; Brightman et al., 1969; Rodbell et al., 1980; Fambrough et al., 1999]. In 1981, Trams et al described nano-sized microvesicles and posed the term exosomes [Trams et al., 1981]. Their experimental design was for the ecto-5'-nucleotidase activity of large microvesicles of size 500-1000nm formed by budding from C6-rat glioma cells. Through electron microscopy (EM) analyses, they found smaller microvesicles which had no ecto-5'-nucleotidase activity and formed in a different pathway from those of the larger microvesicles [Trams et al., 1981]. Later, these microvesicles were shown to be involved in some physiological processes of reticulocyte maturation [Pan et al., 1983]. Starting with those two researches, exosomes have been demonstrated to be important for cellular

functions. Recently, a large body of research has built up showing that exosomes carry signaling molecules which mediate intercellular signaling [Fevrier et al., 2004; Gyorgy et al., 2011]. Moreover, those signaling functions are involved in a variety of cell signaling axes such as neuron-glia signaling, immune system etc., as well as many pathologies, including cancer, which contributes to the complexity of the tumor microenvironment [Fruchbeis et al., 2013; Liu et al., 2006; Kim et al., 2006].

The term exosome can be confused with the intracellular RNA-degrading exosome complex [Mitchell et al., 1997]. However, exosomes are small microvesicles [Johnstone et al, 1987] which are 40-100 nm in size and composed of the same lipid bilayer membrane as the cells they originate from [Stoorvogel et al., 2002]. This feature makes it possible for exosomes to be followed based on the cellular markers they carry. Additionally, knowing the biogenesis of exosomes might shed light on their composition, possible functions and ways of intervention for therapeutic purposes.

Exosomes are thought to be derived from endosomal compartment although, in T Cells, they were demonstrated to be developed by direct budding from the plasma membrane [Booth et al., 2006]. Endosomal system is responsible for the intracellular protein trafficking between cellular organelles and plasma membrane. During endocytosis, early endosome forms via internal budding of the plasma membrane. As part of the maturation process of early endosomes into late endosomes, intraluminal vesicles (ILVs) form within the lumen of the late endosomes, which are then, therefore, called multivesicular bodies (MVBs). ILVs are the potential predecessors of exosomes. During this process, the contents fated to be degraded or exported are sorted into 40-100 nm sized ILVs. MVBs then can either fuse with lysosome for degradation of the contents or can merge back with the plasma membrane for secretion of vesicular contents. [Buschow et al., 2009]. Those released microvesicles are called exosomes (Cocucci et al., 2009). The fusion of MVBs with the plasma membrane and thereby the release of exosomes is an energy-dependent process which also utilizes calcium signaling similar to neurons releasing the packaged neurotransmitters into the axonal cleft (**Figure 5)** [Savina et al., 2003; Savina et al., 2005]. The exosomes, after being released, have similar protein characteristics to the original cell as well as signaling molecules packed in them and can go interact with the target cells upon which they deposit their internal cargo [Thery et al., 2002].

Even though the cellular membrane and the exosomal membrane are similar in content, they are never the same e.g. exosomes lack various cluster of differentiation (CD) and fragment crystallizable (Fc) antigens as well as some integrins that are present on the plasma membrane [Thery et al., 2001]. This shows that, during the channeling of internal budding of the endosomal membrane to form ILVs, some of the proteins are removed or concentrated on the membranes of the future-exosomes. The re-modulation of the membrane content of exosomes is not limited to proteins, but also lipids are subject to removal or concentration [Thery et al., 2001]. For instance, exosomes contain higher levels of ceramide, a lipid with signaling properties [Trajkovic et al., 2008], cholesterol and phospatidic acid (PA) [Laulagnier et al., 2004].

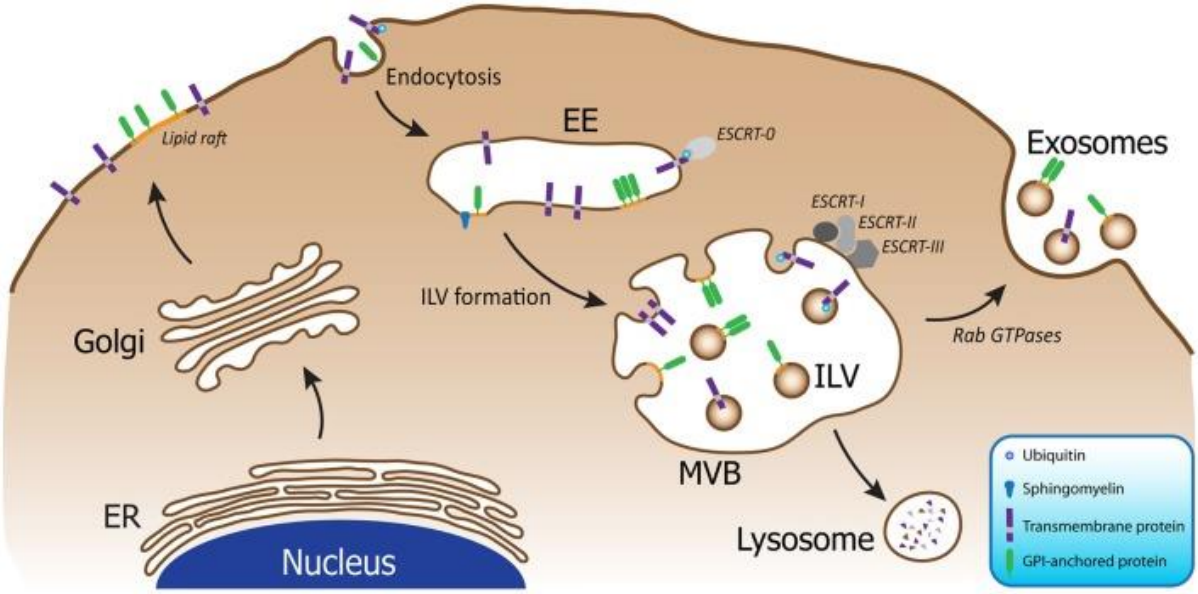


Figure 5: Formation of exosomes. Endocytosis leads to formation of early endosomes (EE). With inward budding of the multi-vesicular body (MVB), intraluminal vesicles (ILV) form. ILVs are packaged with proteins, RNAs, membrane bound receptors, etc. Finally, the MVB fuses with the plasma membrane to release the ILVs as exosomes packaged with relevant signaling molecules. During the packaging of ILVs, ESCRT complexes I, II and III take part, and the exocytosis is mediated by the interplay of Rab GTPases and SNARE complex (Bellingham et al., 2012).

A greatly important process is the sorting of the EV cargo during internal budding of the membrane which leads to ILV formation. The endosomal-sorting complex required for transport (ESCRT) mediates the accumulation and sorting of molecules packed into the ILVs as well as the remodeling of vesicular membranes (Morvan et al., 2012; Adell et al., 2014]. Perturbation of this system leads to defects in the exosomal protein content and the rate of exosome release [Colombo et al., 2013]. Moreover, the process can be adjusted based on the type of the cell and the needs of the cell. For instance, specific RNA-binding ribonucleoproteins (RNPs) on exosomal membranes recognize specific nucleotide sequences on RNAs thereby binding to them as they bud inwards in the late endosome. Which RNAs are packed into the exosomes can be determined by the RNPs. Additionally, reports indicate that, the process of packaging the exosomal cargo involves sphingomyelinase [Laulagnier et al., 2004], Syndecan-Synthenin interaction [Baietti et al., 2012], GTP-binding protein, ADP-ribosyylation factor 6 (ARF6) and its effector Phospholipase D2 (PLD2) [Ghossoub et al., 2014]. At the final stage, where exosomes are released, Rab family of small GTPases [Stenmark et al., 2009; Tauro et al., 2013] and the soluble NSF-attachment protein receptor (SNARE) complex [Fader et al., 2009] have been implicated to play a role.

The exosome content is highly heterogeneous as they may contain proteins, nucleic acids (RNAs such as mRNA and miRNA, DNA) and lipids. Moreover, the protein content of the exosomes is not only the intravesicular proteins but also the surface molecules [D'Asti et al., 2012]. The exosome cargo is precisely modulated by an interplay of a variety of regulatory mechanisms governing the type and physiological condition of the donor cell, the stimuli causing the exosome production and the pathways leading to production of different exosome types [Minciacchi et al., 2015]. Those processes also make the exosome function broad, based on the contents. Exosomes contain integrins which are important for the interactions with extracellular matrix (ECM) and for the interaction with the recipient cell, leading to internalization [Rieu et al., 2000; Ngora et al., 2012]. Moreover, major histocompatibility complexes (MHC) have been shown to be present on exosomal membranes where they can contribute to antigen presentation in case of an infection or a cancer [Testa et al., 2010; Yang et al., 2012]. Some other proteins such as Alix, Tsg101, flotillin, Rab, and tetraspanin family members of proteins are found on exosomes and are also used as exosome markers. Those proteins are also involved in exosome biogenesis [Colombo et al., 2013; Romancino et al., 2013; Trajkovic et al., 2008; Savina et al., 2002; Ostrowski et al., 2010]. Among those tetraspanins, especially CD9, CD63 and CD81 have been shown to be involved in the endosomal vesicle trafficking (**Figure 6)** [Pols and Klumperman, 2009; Abache et al., 2007].

Intracellular compartment of the exosomes also contains multiple proteins and functional genetic elements (**Figure 6**). The proteins are packaged via the interactions with the exosome biogenesis machinery [Biaetti et al., 2012]. Additionally, tetraspanins interact with the membrane proteins in a direct manner or via entrapment into tetraspanin microdomains mediate their loading into the exosomes [Mazurov et al., 2013]. The protein content in the intravesicular compartment is mostly dependent on the cell of origin, however, glyceraldehyde-3-phosphate dehydrogenase have been shown to be included in many types of exosomes which points out for a role for exosomes in metabolism [Tisdale et al., 2001; Ronquist et al., 2013]. Apart from proteins, exosomes were shown to harbor RNAs and transfer them between cells [Ratajczak et al., 2006]. Subsequently, it was demonstrated that the mRNA transferred from one cell to another via exosomes can be translated into protein [Valadi et al., 2007]. Moreover, exosomes released from malignant tumors have been shown to transfer a malignant phenotype to formerly less aggressive tumors [Zomer et al., 2015]. Apart from the mRNAs, exosomes also are rich in small non-coding RNA species through which they function to modulate the gene expression in the recipient cells [Baglio et al., 2015; Koppers-Lalic et al., 2014]. Those small non-coding RNAs consists mostly of miRNAs which regulate the gene expression at the post transcriptional level in the recipient cell upon transfer via exosomes (**Figure 5**). They bind to the 3' UTR regions of the mRNAs and either direct them to degradation or prevent their translation by staying bound to the mRNA thereby causing abortion of translation by the ribosomal machinery. Evidence suggested that oncogenic miRNA transfer from one tumor cells to a healthy cell causes a malignant transformation whereas, tumor-suppressive miRNA transfer into a tumor cell caused regression of tumor properties [Aucher et al., 2013; Kruger et al., 2014]. Whether specific miRNAs are loaded into exosomes and if so, what mediates the selective loading of these miRNAs, however, is still unclear. It is important to uncover the contents of exosomes in order to reveal their true roles

in the cellular and tumor biology. Therefore, the accumulating research findings have been compiled into online databases which are available for scientific use. Those databases are: Exocarta, Vesiclepedia and EVpedia [Mathivanan et al., 2009; Kalra et al., 2012; Kim et al., 2013].

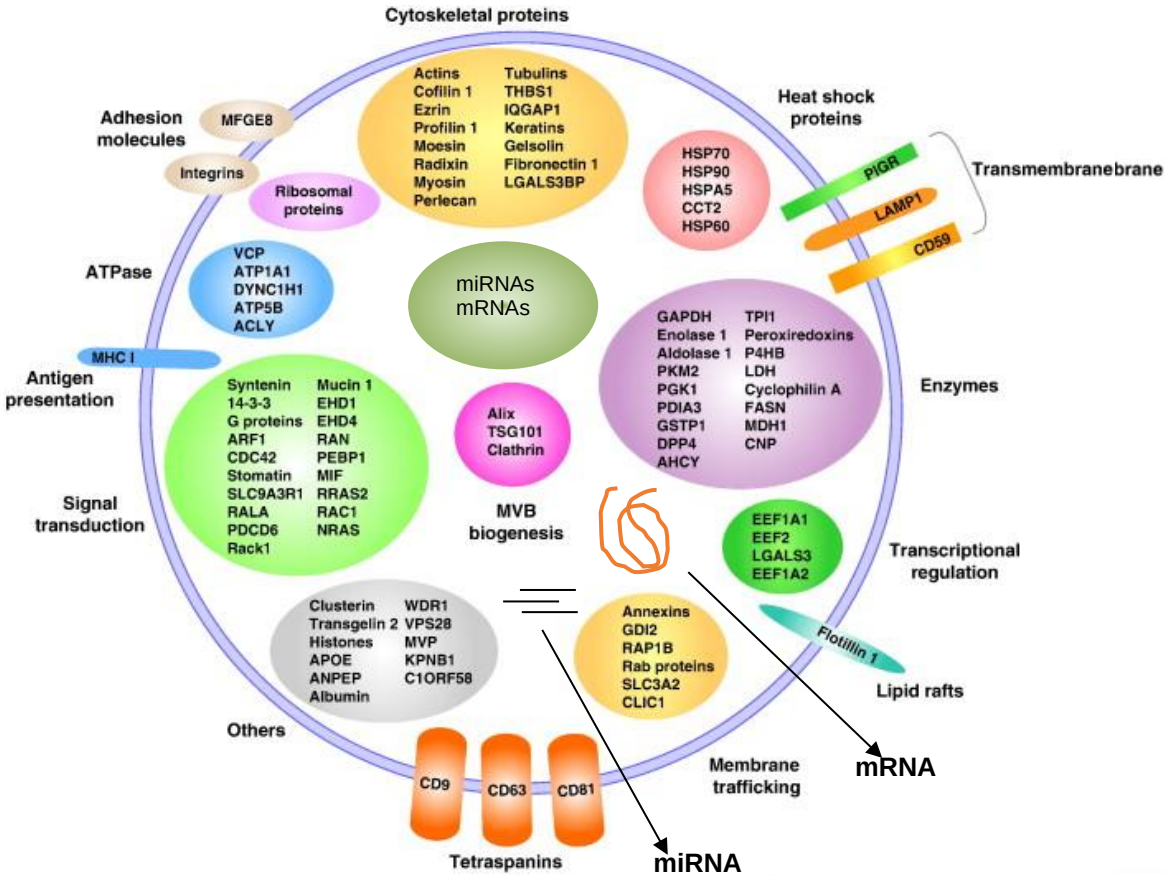


Figure 6: Structure and the cargo of an exosome. Exosomal cargo includes many membrane-bound proteins such as tetraspanins, target cell recognition antigens, adhesion molecules, as well as intravesicular elements involved in cargo packaging and mRNAs, miRNAs and enzymes [image modified from Mathivanan et al., 2010].

Exosomes interact with the recipient cell in four different defined ways: direct fusion and deposition of the contents into the recipient cell, receptor-mediated internalization (upon receptor interaction on plasma membrane or on lipid rafts), receptor activation via interaction of exosome membrane-bound ligand with a receptor on the cell surface of the recipient cell and antigen presentation which mostly occurs in the immune system (**Figure 7**) [Denzer et al., 200; Thery et al.,2011]. As mentioned above, exosomal membranes contain specific proteins. Those proteins direct exosomes to certain cell types by acting as interaction partners for the proteins on the recipient cells. This means exosomes are not randomly taken up by any cell in

the vicinity, but in fact they are destined to specific cell types, which adds up into the complexity of exosome biology. Upon interaction of the exosome-surface proteins with the recipient cell-surface proteins, exosomes either release their contents into the cytosol of the recipient cell or are taken up via receptor-mediated endocytosis. In both cases, the exosomal membranes, and so the proteins, are integrated into the membrane of the recipient cell. However, the exosomes which are taken up via receptor-mediated endocytosis sometimes might be targeted for degradation. That is why, only the exosomes escaping those degradation processes can mediate an effect in the recipient cell [Svensson et al., 2003; Prada and Meldolesi et al., 2016].

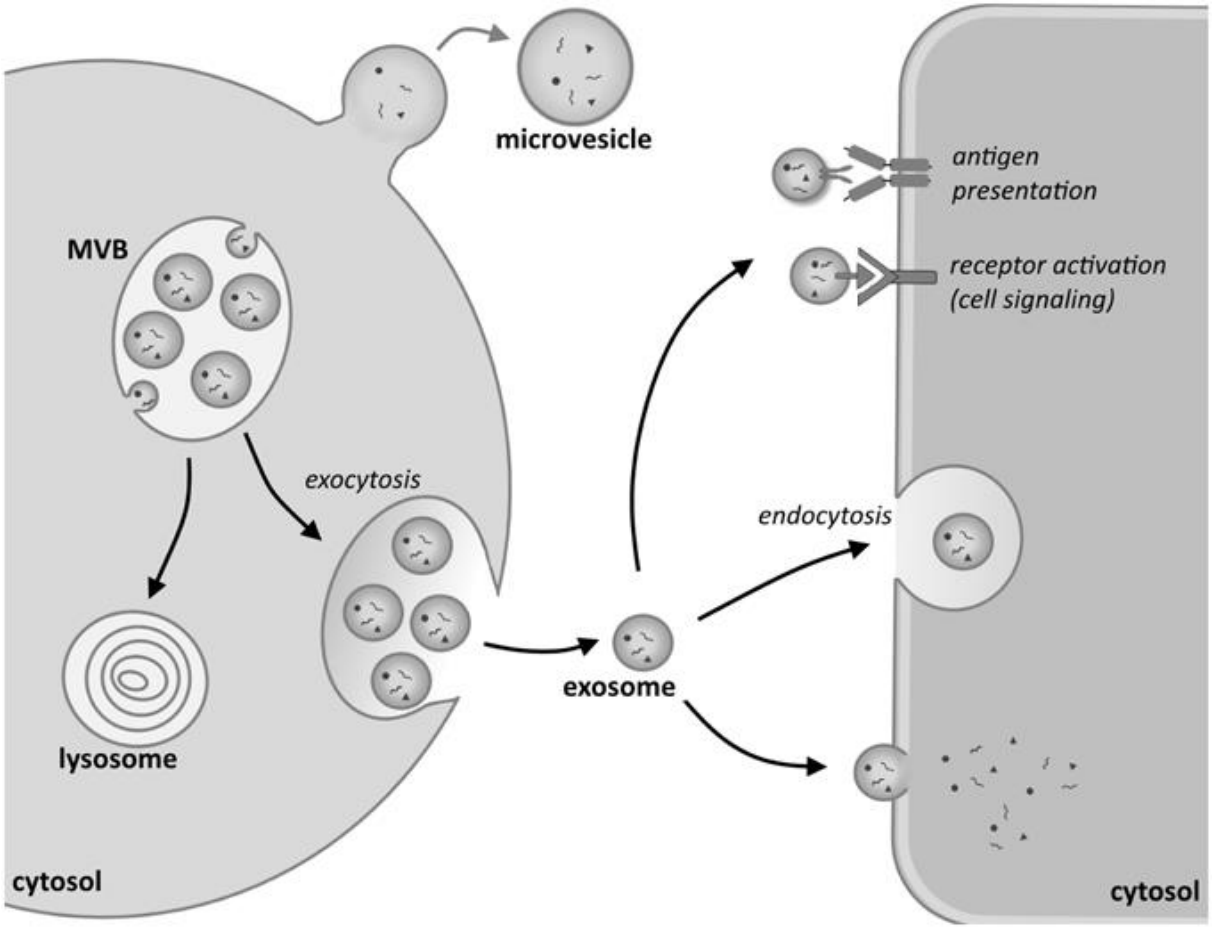


Figure 7: Exosomes interact with the target cells in several ways. They can (I) act for antigen presentation in the immune system, (II) activate cell surface receptors on the recipient cell by the binding of the membrane-bound ligands on exosomes, (III) be taken up by endocytosis and (IV) fuse directly with the plasma membrane of the recipient cell depositing the contents into the cytosol [Krause et al., 2015].

1.5. Gliomas and Exosomes

Exosomes were first identified in cancer, and subsequently, researchers invested large efforts into uncovering the roles of exosomes in cancer research. Within various experimental settings, researchers defined many roles for exosomes in cancer. For instance, exosomes from melanomas induce molecular alterations in the malignant tumor cells thereby mobilizing them to leave the initial tumor location, which in turn contributes to metastatic dissemination. Additionally, those exosomes prepare a metastatic target niche in the sentinel lymph node for those mobile cells by increasing the production of ECM components. Exosomes also stimulate proangiogenic factors which eventually results in nodal metastases (Hood et al., 2011). Moreover, exosomes decrease the immune surveillance via enhancing the levels of immunosuppressive cells, decreasing the proliferative capacity and cytotoxic properties of NK and T Cells as well as the number and function of APCs [Zhang et al. 2011; Zhang et al., 2012]. Last but not least, exosomes are utilized as mechanisms of chemoresistance in tumors where they are packed with the chemotherapeutic agent and exported out of the cell which is called vesicle shedding [Shedden et al., 2003]. In contrast to those findings in tumors, less extensive research has been done on gliomas.

The exosomes in the normal brain are released both by neurons and glia to rapidly signal between each other to mediate protective effects against stress, to promote repair, survival and immunity as well as synapse and plasticity formation [Budnik et al., 2016]. In brain tumors, those properties of exosomes are hijacked in order to increase the growth and adapt to changes in the microenvironment. The exosomes released from GBMs have been shown to include mRNAs and miRNAs [Skog et al., 2008]. Those RNA species are selectively packaged into exosomes, as opposed to the parental cell, for specific purposes based on the conditions [Li et al., 2013]. In line with this, glioma cells under hypoxic conditions have been shown to increase the hypoxia-related mRNA and protein content in exosomes they released [Kucharzewska et al., 2013]. Moreover, GBM-derived exosomes have been demonstrated to contain oncogene protein products such as epidermal growth factor receptor variant III (EGFRvIII). This growth factor receptor is constitutively active and accumulates on the lipid rafts of the recipient cells, where it starts a signaling cascade to eventually alter the gene functions related to the growth via ERK1 – ERK2 and AKT pathways [Al-Nedawi et al., 2008]. Treps et al showed that, exosomes also transfer the vascular permeability factor Semaphorin 3A to the adjacent endothelial cells which elevates the vascular permeability favoring the tumor angiogenesis [Treps et al., 2016]. Furthermore, GSC-derived exosomes contain TGF-β which inhibits the immune response of the recipient immune cells in the vicinity such as microglia and macrophages/monocytes by altering their cytokine expression profile [Graner et al., 2009]. The exosomes, however, not only contain pro-tumorigenic elements but also may contain anti-tumorigenic species such as miR-1. miR-1 targets Annexin – A2 mRNA, which is one of the most abundant mRNAs in the GBM-derived exosomes and whose protein product is pro-ongogenic [Bronisz et al., 2014].

The findings of the exosome studies have been used for further translational research. Wolfers et al demonstrated that the glioma-derived exosomes carry antigens specific to the tumor. Those exosomes were then used to potentiate dendritic cells which in turn activated T-cells causing a massive cytotoxicity [Wolfers et al., 2001]. This discovery made the researchers

question if tumor-derived exosomes could be used as vaccines to immunize the patients. However, there has been some adverse effects reported regarding the use of exosomes in tumor therapy [Zhang et al., 2011; Zhang et al., 2013]. Additionally, it was shown that, upon treatment with ionizing radiation, glioma exosomes gain increased levels of some growth factors and growth factor receptors. Those exosomes then induced activation of tyrosine kinase receptor type 1 (TrkA) and a well-known proto-oncogene Src kinase in non-irradiated glioma which altogether lead to an increase in the migration and invasion showing that irradiating gliomas cause enhanced migration and invasion of the surrounding tissue by the non-irradiated cells of the tumor [Arscott et al., 2013]. As for the diagnostic purposes, Skog et al demonstrated that, GBM patient sera contained EGFRvIII mRNA and miR-21 in the exosomes as opposed to those from the healthy donor sera. This finding then brought the notion that the circulating exosomes could be used to identify the mutation status as well as the gene expression profile of the brain tumors [Skog et al., 2008]. However, the available miRNA copy numbers were relatively low, making the analysis difficult. Akers et al then demonstrated that the spinal fluid could be used for the same purpose where the copy numbers are high enough for further analyses [Akers et al., 2015]. All in all, the fact that the exosomes in the bodily fluids could be used for diagnostic purposes for brain tumors have been speculated as a promising tool, because they can be easily collected without any invasive procedures as in biopsies; they contain valuable information regarding the genetic status of the tumor cell they originate from; and the half-life of them are short in the body fluids which can be an advantage for monitoring the short-term changes in the tumor cells [André-Grégoire et al., 2017].

Exosomes have been also exploited to deliver anti-tumorigenic species into the tumor tissues because they can travel through body fluids, cross membranous structures such as BBB and easily access the brain. In one study, exosomes were made to contain a peptide on their surface which can bind to EGFR. Those exosomes were packed with let-7a miRNA targeting EGFR and administered intravenously in an EGFR-expressing breast cancer model. Exosomes were shown to reach the tumor cells and inhibit tumor growth [Ohno et al., 2013]. In another study, exosomes were shown to reach the brain via intranasal application. Moreover, exosomes that were packed with curcumin had significant effects on the delay of the tumor growth in GL261 mouse tumor model [Zhuang et al., 2011]. Moreover, exosomes have been engineered to deliver synthetic vectors, viral Exosomes, therefore, could be utilized as vehicles to deliver anti-tumorigenic agents into tumor cells upon engineering for specific purposes.

1.6. MSCs, Exosomes and Tumors

Exosome research in the context of tumors, and specifically gliomas, have mostly focused on the tumor derived exosomes. However, tumors are complex structures that are in close relationship with the microenvironment. Therefore, it is imperative to uncover the effects of the exosomes from the vicinity on gliomas. There are many cell types in the tumor microenvironment, some of which have been explained above in the context of brain tumors. One cell type proven to be important, which is also the main focus of the thesis, is the MSCs.

MSCs have been implicated in studies regarding exosomes in the context of tumor. BMSCs have been shown to contain, in addition to classical exosome markers such as Alix, Tsg101,

CD9, CD63 and CD81, PDGFR-β, TIMP-1 and TIMP-2 which support tumor growth [Vallabhaneni et al., 2015]. Additionally, some proteins which are implicated in the packaging and transport of RNA, such as argonaute 2, Staufen1 (Stau1) and Staufen2 (Stau2), have been shown to be present in the exosomes of MSCs [Collino et al., 2010]. Moreover, some proteins which promote angiogenesis via Wnt – β-catenin pathway are also carried in the exosomes derived from MSCs [Chen et al., 2014]. In addition to those protein cargos, exosomes from MSCs carry also mRNAs whose protein products are implicated in the control of cytoskeletal reorganization, regulation of transcription, cell immunity and differentiation as well as proliferation and cellular growth [Bruno et al., 2009; Tomasoni et al., 2013]. Moreover, some miRNAs in the form of pri-miRNAs are present in MSC-derived exosomes some of which are related to promoting cellular growth and preventing apoptosis [Katakowski et al., 2013] as well as decreasing tumor growth [Xin et al., 2013].

As a result of the identification of exosome cargos of MSCs, they have been identified as important contributors of tumor pathology in the microenvironment. In addition to previously-mentioned pro- or anti-tumorigenic effects on tumors that are mediated via secreted species or direct contact, MSCs also have been shown to exert profound effects on tumors via their exosomes. It was shown that, MSC-derived exosomes increase VEGF expression which in turn favors the tumor growth via ERK1/2 and p38-MAPK pathway. This situation was reversed by ERK1/2 inhibition [Zhu et al., 2012]. Moreover, MSC-exosomes promoted metastasis, proliferation and survival of myeloma cells by influencing the activities of p38, p53, c-Jun N-terminal kinase and Akt pathways [Wang et al., 2014]. Yang et al demonstrated that the stimulated via MSC-derived exosomes, tumors gain new cellular dynamics which are beneficial for the re-organization of the microenvironment [Yang et al., 2015]. Interestingly, those exosomes of MSCs demonstrated the capability of transferring mRNA for a growth factor into tumor cells where it induced ERK1/2 – MAPK pathways thereby promoting the transition of cell cycle from G0/G1 to S phase [Du et al., 2014]. MSC exosomes not only enhance tumor growth but also promote chemoresistance. MSC-exosomes have been shown to boost resistance against bortezomib used in multiple myeloma treatment [Wang et al., 2014] by preventing apoptosis [Ono et al., 2014].

Contrary to those pro-tumorigenic properties, MSC-derived exosomes have anti-tumorigenic effects too. They block tumorigenesis by preventing the cell cycle progression in hepatocellular carcinoma, Kaposi's sarcoma and a type of ovarian cancer by upregulating expression of some genes which favor a dormant cell state. They also induced apoptosis in Kaposi's Sarcoma and hepatocellular carcinoma cells [Bruno et al., 2013]. Moreover, exosomes from MSCs blocked the expression of VEGF in mouse breast cancer cells thereby inhibiting angiogenesis [Lee et al., 2013]. The mechanisms of antitumor capabilities of MSC – derived exosomes have not yet been fully characterized, therefore contains large gaps in the information pool. And in terms of MSC-exosomes and glioma interaction, there isn't a large body of research. Recently, Figueroa et al demonstrated that, Glioma-associated exosomes increase the proliferation and clonogenicity of GSCs and carry miR-1587 which inhibit nuclear receptor co-repressor 1 (NCOR1), a tumor suppressor [Figueroa et al., 2017]. Moreover, Munoz et al utilized MSCs as delivery vectors for anti-miR-9 whose transfer was mainly achieved via exosomes. They demonstrated the chemosensitization of GBMs by inhibiting

miR-9 which is important for acquisition of resistance to TMZ by being involved in the expression of P-glycoprotein, a drug efflux transporter [Munoz et al., 2013]. One interesting study utilized the intratumoral injection of exosomes from miR-146 expressing MSCs. In this setting, exosome-laden miR-146 inhibited translation of EGFR reducing growth and invasion capacities [Katakowski et al., 2013]. In addition, GBM-derived exosomes have effects on MSCs as well [Chowdhury et al., 2015] making the interaction between MSCs, the exosomes released from MSCs and the GBMs very complex.

1.7. Summary

All in all, gliomas are therapy-resistant, highly aggressive and deadly cases. One major cause of the incurability is brought about by the intimate relationship of glioma cells with the tumor microenvironment, which supports the tumor growth in many ways by providing a permissive neighborhood. In the tumor microenvironment are BTSCs, ECs and hypoxic regions, immune cells and immune modulatory cues, astrocytes, neural stem/precursor cells and MSCs. Different cell types contribute to tumor pathology in different ways. One eminent and not-yet-fully-explored interaction is the one between MSCs and GBMs. MSCs have pro-and anti-tumorigenic effects on GBMs and the quality of this interaction is dependent on the source of MSCs and the way interaction takes place. Exosomes are minute vesicles carrying signaling materials which are secreted by many cell types in the tumor microenvironment. However, exosomes from GBM cells and the MSCs are of utmost importance as the tumor-derived exosomes hijack the surrounding to permit growth of the tumor and MSC-derived exosomes mediate supportive or suppressive effects on tumors. While the latter case has not been explored very deeply, the effects of tumor-derived exosomes on the microenvironment are well-established. That is why, it is vital to shed light on the interaction mechanisms of MSCs and MSC-derived exosomes with GBMs, which may support therapeutic approaches by providing a novel treatment target. Furthermore, it might be a base for further studies which utilize MSCs or MSC-derived exosomes as vectors for tumor therapy.

2. AIM OF THE THESIS

MSCs have been shown to be at the interplay of many pathologies including cancer (Gottschling et al., 2013; Lamb et al., 2005; Santamaria-Martinez et al., 2009; Wallace et al., 2001; Dvorak et al., 1986). Although certain roles have been assigned to MSCs about their homing behavior to cancers in general, the studies as to what roles MSCs play in the brain tumor niche are very few. Moreover, the interaction between MSCs and the GBM cells is claimed to be both pro-tumorigenic and anti-tumorigenic, and under what circumstances they behave one way or another is largely unexplored. Therefore, shedding light on this interaction might prove beneficial for developing treatment strategies against these devastating disorders.

The aim of this study was to explore the mode of interaction between MSCs and GBMs, and to determine relevant signaling pathways.

Specific questions are:

1. Under what circumstances do MSCs act as pro- or anti-tumorigenic?
2. Are pro- or anti-tumorigenic conditions more relevant to the *in vivo* interaction between MSCs and GBMs?
3. What pro-tumorigenic impacts do MSCs have on GBMs?
4. What are the modes of signaling between MSCs and GBMs mediating either pro- or anti-tumorigenic effects?
5. What signaling molecules are involved in pro-tumorigenic pathways induced in GBMs by MSCs?
6. Do MSCs render GBMs chemoresistant? What molecules take part in chemoresistance induction?
7. Can we generate a universal mouse model to study the interaction of MSCs with various GBM subtypes for *in vivo*?

3. MATERIALS AND METHODS

3.1 List of Some Basic Materials

3.1.1. Cell Culture Contents

Material	Supplier	Cat #
Dulbecco's Modified Essential Medium (DMEM)	Biochrom GmbH	FG0415
DMEM F/12	Thermo Fischer Scientific	11320033
NeuroCult™ Basal Medium	Stemcell Technologies	05700
Roswell Park Memorial Institute Medium (RPMI)	Thermofischer Scientific	11875093
NeuroCult™ Proliferation Supplement	Stemcell Technologies	05701
Fetal Calf Serum (FCS)	Thermofischer Scientific	10270106
B27	Thermo Fischer Scientific	17504044
Minimal Essential Medium Non-essential Aminoacids (MEM NEAA)	Thermo Fischer Scientific	11140068
Pen/Strep	Thermo Fischer Scientific	15140122
hEGF	Thermo Fischer Scientific	PHG6045
FGF	Thermo Fischer Scientific	PHG0263
Trypsin	Biochrom GmbH	L 2123
Accutase	Stem Cell Technologies	07920

3.1.2. Cell Culture Materials

Material	Supplier	Cat #
Cell Culture Flasks (T25; T75; T150)	TPP	90026; 90076;90156
Well-Plates (96, 24, 12, 6)	TPP	92696; 92424; 92412; 92406
Falcon Tubes (15 ml; 50 ml)	TPP	91015, 91051
Microfuge Tubes	Eppendorf	T9661-1000EA
Countess II FL	Thermo Fischer Scientific	

3.1.3. Reagents Purchased

Material	Supplier	Cat #
Non-Radioactive Cell Viability/Proliferation Assay	Promega	G4100
Temozolomide (TMZ)	Sigma Aldrich	T2577
QuantiTect Reverse Transcriptase	Qiagen	205310
RNeasy RNA Isolation Kit	Qiagen	74106
ExoQuick-TC™ Exosome Isolation Kit	System Biosciences	EXOTC50A-1

3.2 TaqMan Assays

3.2.1 QKIs (Thermo Fisher Scientific)

QKI-5: Hs00916681 (RefSeq: NM 006775.2)

QKI-7: Hs00920546 (RefSeq: NM 206854.2)

QKI-general: Hs00916678 (RefSeqs: NM 006775.2; NM 206853.2; NM 206854.2)

For Calculation of QKI-6 amounts, I subtracted the amounts of QKI-5 and QKI-7 from QKI-general results.

3.1.2. EFNA3

EFNA3: Mm01212723_g1 (RefSeq: NM_010108.1)

3.3. Vectors for Overexpression and Knockdown

Overexpression and knockdown vectors were all purchased from BioCat.

3.3.1. Knockdown

QKI: TLHSU1400-9444-pZIP-mCMV-ZsGreen-GVO-TRI

EFNA3: TLMSU1400-13638-pZIP-mCMV-ZsGreen-GVO-TRI

Non-targeting Control: TLNSU-1400-GVO-TRI

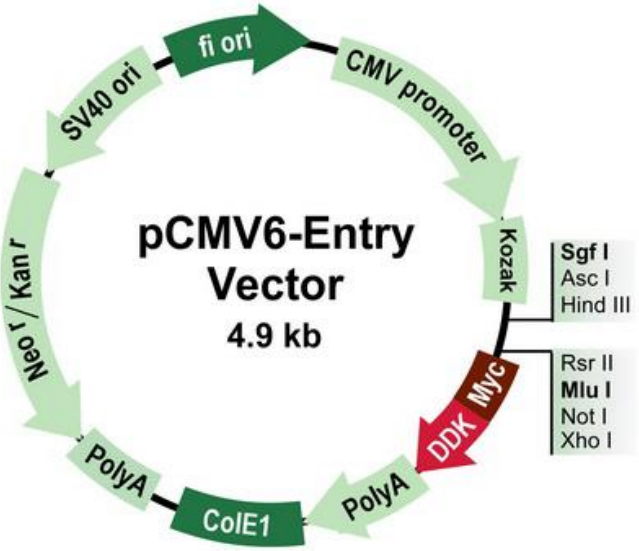
3.3.2. Overexpression

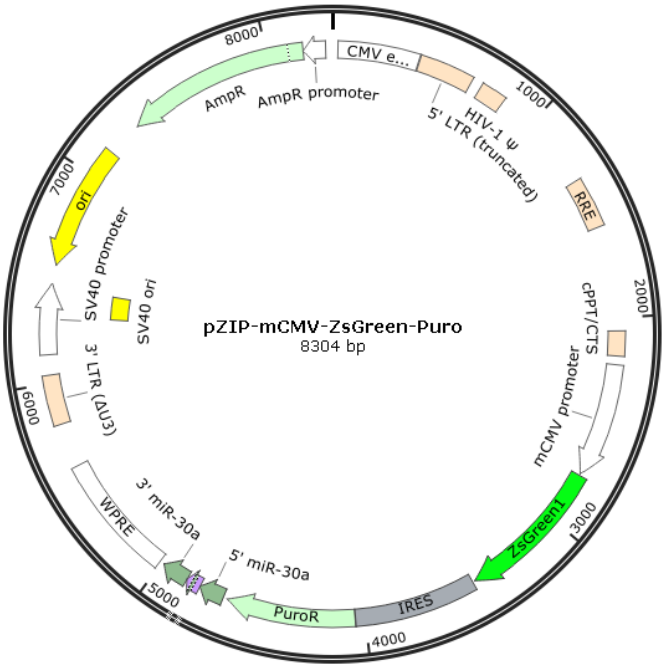
QKI-5: RC205779-OR

QKI-6: RC224090-OR

QKI-7: RC215734-OR

EFNA3: BC107002-TCM1004-GVO-TRI


pCMV6-Entry
Vector
4.9 kb
fi ori
CMV promoter
Kozak
Sgf I
Asc I
Hind III
Rsr II
Mlu I
Not I
Xho I
Myc
DDK
PolyA
ColE1
PolyA
Neo r / Kan r
SV40 ori


pZIP-mCMV-ZsGreen-Puro
8304 bp
8000
CMV e...
AmpR
AmpR promoter
1000
HIV-1 ψ
5' LTR (truncated)
RRE
2000
cPPT/CTS
mCMV promoter
ZsGreen1
3000
IRES
PuroR
4000
5' miR-30a
3' miR-30a
WPRE
5000
3' LTR (ΔU3)
6000
SV40 ori
SV40 promoter
ori
7000

3.4. Cell Culture

Medium	Cell Line
DMEM F/12 with B27 (1X), Pen/Strep (1%), human Embryonic Growth Factor (hEGF) and Fibroblast Growth Factor (FGF). (10 ng/ml each)	GBM10
	GBM13
	GBM14
	GBM20
	NCH588J
	NCH684
	NCH421k
	NCH644
	NCH592b
	P53KO-PDGFB-GFP
NeuroCult™ Basal Medium supplied with NeuroCult™ Proliferation Supplement, Pen/Strep, hEGF and FGF (10 ng/ml each)	Line 4
	Line 6
	Line 7
	Line 8
	Line 9
	Line 11
DMEM, supplied with 20% FCS, MEM NEAA (1%) and Pen/Strep (1%)	MSC
RPMI supplied with 10% FCS and Pen/Strep (1%)	HEK293
DMEM, supplied with 10% FCS, MEM NEAA (1%), Pen/Strep (1%)	HEK293T
	GL261

3.4.1. MSC Cell Culture

Bone marrow derived MSCs were purchased from Thermofischer Scientific (Cat. #: S1502100) and cultured according to manufacturer's instructions. Briefly: MSCs were maintained in Dulbecco's Minimum Essential Medium (DMEM – Biochrom, Cat #: FG0415) supplied with 20% FCS (Thermofischer Scientific - 10270106), 1% MEM NEAA (Thermo Fischer Scientific – Cat #: 17504044) and 1% Pen/Strep (Thermo Fischer Scientific – Cat #: 15140122) with a seeding density of 300,000 cells/150 cm^2 and grown at 37 ˚C under 5% CO_2/95% humidified air. While passaging, 20% FCS medium is discarded and the cells were washed with PBS of appropriate volume based on the size of the culturing flask (3 ml for T25; 6 ml for T75 and 10 ml for T150) and trypsin (Biochrom GmbH – Cat #: L 2123) is added again based on the size of the culturing flask (1,5 ml for T25; 3 ml for T75 and 6 ml for T150) and the flask is incubated for 3-5 mins at 37 ˚C until the cells dissociate. The trypsin is then inactivated by addition of DMEM containing 20% FCS and the MSCs are split. The passaging is carried out every 2 days.

3.4.2. GSC Cell Culture

GSC primary cultures were kept either in DMEM/F-12 or NeuroCult™ Basal Medium (mouse). Both cultures were maintained at 37 ˚C under 5% CO_2/95% humidified air. GSCs grow in form of spheroids. When passaging, GSC cell suspension is taken into falcon tubes and centrifuged at 400xg for 5 mins. The supernatant is discarded and the spheroids are either resuspended in 1 ml of fresh culture medium and dissociated with rigorous pipetting with a 1 ml – micropipette or when the spheroids are firm, they are resuspended in 1 ml of accutase, a cell detachment solution (Stem Cell Technologies – Cat #: 07920), and dissociated with rigorous pipetting with 1 ml – micropipette. When accutase is used, the GSCs are washed with addition of 9 ml of cell culture medium and additional centrifugation of 5 mins at 400xg. The supernatant is discarded and the GSCs are split at an appropriate seeding density specific for each GSC line determined based on their growth rate. The passaging is carried out every 3 days. All GSCs were maintained in normal cell culture flasks, except p53KO-PDGFB-GFP, which was cultivated in normal microbiology-grade petri dishes due to high potential of adherence and differentiation in coated cell culture dishes.

3.4.3. HEK293 Cell Culture

HEK293 cells were cultivated in RPMI (Thermofischer Scientific, Cat #: 11875093) supplied with 10% FCS, 1% MEM NEAA and 1% Pen/Strep. These cells are also adherent, similar to MSCs, so the passaging protocol was the same as that of MSCs.

3.4.4. Freezing Medium

In order to keep stocks of low passage cells, I froze aliquots at low passages at -80 ˚C until they were needed. For this, I used a special type of medium for different cell types. For GSCs, it included (at final mixture with cells included) 5% DMSO in DMEM/F-12 without additives. For MSCs, it included (at final mixture with cells included) 5% DMSO and 10% FCS in DMEM. The concentration of DMSO and FCS were double in the stock solutions of freezing medium. When I freeze cells, I mix 500 µl of cell suspension in respective medium (without additives) with 500 µl of the appropriate freezing medium. The cells were then placed into isopropanol-submerged cryovial holders and placed into -80 ˚C for overnight. Then cryovials were taken into liquid nitrogen tank for long term storage.

3.4.5. Cell Counting

I counted cells throughout my experiments both manually and with a cell counter. For the manual cell counting, I centrifuged the cells and discarded the supernatant. Then I resuspended the cells in 1 ml of culture medium, mixed 20 µl of cell suspension with 20 µl of trypan blue and placed approximately 20 µl of the mixture into cell counting grid (Neubauer). For the automated cell counting, I used the same cell suspension – trypan blue mixture with a special counting grid and inserted the grid into counting machine (Countess II FL from Thermo Fischer Scientific). This device counts the cells and calculates the amounts of cells in 1 ml of medium.

3.5. Conditioned Medium (CM)

MSCs were plated in dishes with 150 cm^2 culturing area at a density of 300,000 cells per dish and maintained in normal culturing conditions (DMEM with 20% FCS) at 37 ˚C under 5% CO_2/95% humidified air. After 2 days when they reached a near-confluent stage, the growth medium was discarded, the cells were washed with a large amount of PBS (20 ml) and DMEM with no FCS (but 1% Pen/Strep and MEM NEAA) was added. After cultivating for 72 hours, the suspension medium was harvested by centrifuging the cells and cell debris at 3000xg for 15 mins and filtering with a pore size of 0.45 µm.

3.6. Viability Assay

Non-Radioactive Cell Viability/Proliferation Assay was purchased from Promega (Cat #: G4100) and applied based on manufacturer's instructions. Briefly: 7,000 cells were plated in each well of a 96-well plate (5 replicates for each condition and for each cell line used), either with CM or with control (DMEM without FCS) for 72 hours. On day 3, pictures of the cells in each well were taken for additional analyses. Then dye solution from the viability assay was added, 15 µl in each well and the plates were incubated at 37 ˚C under 5% CO_2/95% humidified air for 4 hours. Then, stop/dissociation solution of 100 µl was added in each well to stop the assay, disrupt the cells and dissolve the purple oxalate crystals formed within the cells to produce a colored solution. The plates are then incubated again in the same incubation conditions as before for 1 hour. The colorimetric absorbance was then measured by elisa reader aided by a computer. The background-subtracted absorbance values are then analyzed using Nalimov's Test in order to determine the outliers. The absorbance of the control cells (cells maintained in normal cell culture medium, DMEM) were averaged (from 5 replicates) and the average value was arbitrarily set as 1. The same averaging was performed for experimental conditions and they were normalized to the average of control cells. The values were then plotted as bar graphs.

3.7. Chemoresistance Assay

TMZ (25 mg) was dissolved initially in 644 µl of DMSO and then further dissolved in DMEM to reach 200 mM stock solution in order to prevent DMSO cytotoxicity. From that stock it was diluted to 100 µM or 300 µM either in DMEM or CM.

In a 6-well plate format, 100,000 cells per well with 3 replicates of DMEM (Control) or CM with 100 or 300 µM TMZ in DMEM or CM were plated. For the consecutive days, the media in each well was replaced with fresh media with or without TMZ in DMEM or CM. After 3 days of TMZ treatment, the cells were moved into their regular culturing medium. On day 7, the cells were counted and 100,000 cells were plated back for counting on day 14. On day 14, the same way, cells were counted and 100,000 cells were plated again. On day 21 the final count was performed and cells were discarded (For an illustration of the method applied, see the figure below). The calculation was performed based on the total amount of cells on each counting day, i.e. 100,000 cells were plated back but the calculation was made based on the total number of cells counted on that specific day. The results were then plotted.

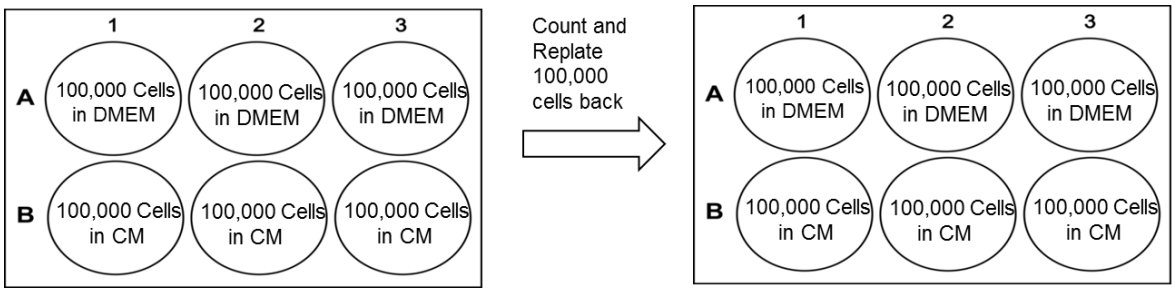


Figure 1: Method used for chemoresistance assays. 100,000 GSCs were initially plated with or without TMZ in either CM or DMEM. The media were exchanged consecutively with fresh media with or without TMZ in either DMEM or CM. On day 7, 14 and 21, the cells were counted and 100,000 cells were plated back into the same well for day 7 and 14. On day 21, the final count was performed and the GSCs were discarded. The cell number on day 14 and 21 was calculated by assuming that I plated all the cells, instead of 100,000.

3.8. Exosome Isolation and Depletion

Exosomes were isolated via ExoQuick-TC™ Exosome Isolation Kit for Cell Culture Media (System Biosciences, Cat #: EXOTC50A-1) from Promega following manufacturer's instructions. Briefly, 2 ml of ExoQuick-TC was mixed with 10 ml of CM (see above for the production of CM), mixed by inverting a few times and incubated overnight at 4 ˚C. Then the mixture was centrifuged at 2000xg for 40 min. The supernatant was discarded and the pellet was centrifuged for an additional 5 mins to get rid of culture media completely, and the resulting pellet was dissolved in DMEM.

In order to deplete the exosomes from CM, I used an ultracentrifuge. I collected the CM freshly, centrifuged to eliminate cell debris and filtered in the same way as explained above, and centrifuged the CM overnight at 100,000xg. The pellet was then discarded and the exosome-free supernatant was then used for further experiments.

3.9. Transfection, Virus Production and Transduction

For overexpressing the QKIs, I used pCMV6-Entry Vectors from BioCat (see below for a detailed map of the vector) and transfected the GBMs. Briefly: 0,5 µg of plasmid, pCMV-6, was diluted in a total volume of 25 µl of Opti-MEM Reduced Serum Media (Thermofischer Scientific, Cat #: 31985062). 1 µl of OMNIfect Transfection reagent (transOMIC, Cat #: OTR 1001) was also diluted Opti-MEM reduced serum medium and those two mixtures were mixed together. The mixture was incubated for 10 mins at room temperature. The mixture was then added onto 500 µl of cell suspension, and the cells were incubated at 37 ˚C under 5% CO_2/95% humidified air for 3 days. pCMV-6 vector contains a neomycin resistance cassette. Therefore, the transfected cells were selected via G418 application. The concentration of lethal doses of G418 was determined for each GSC line separately, previously. The G418 selection was carried out for 2 weeks and cells were then assayed further.

The knockdown was achieved by shERWOOD-UltramiR-Lentiviral constructs – pZIP-mCMV-ZsGreen-Puro (see above for a detailed map of the lentiviral vector) through stable

transduction. Using these vectors, I first produced lentiviral particles. I used the TransLenti Viral GIPZ Packaging System (from Thermo Scientific, Cat #: TLP4615) for this. I plated 5,5x10^6 cells of HEK293T cells in a T75 tissue culture flask in DMEM with 10% FCS, 1% MEM NEAA and 1% Pen/Strep. I mixed 9 µg of lentiviral vector with 28,5 µg of TransLenti Viral Packaging mix in 1 ml of DMEM with no additives, mixed it with 187,5 µl of Arrest-In Transfection reagent dissolved in 1 ml of culture medium with no additives and incubated for 20 mins at room temperature. Then I aspirated the culture medium from the flask and added 2 ml of the transfection mix and completed the medium to 5 ml with addition of plain DMEM. The cells were then incubated at 37 ˚C under 5% CO_2/95% humidified air for 6 hours. I then aspirated the transfection medium and added 10 ml of normal growth medium, DMEM with 10% FCS, 1% MEM NEAA and 1% Pen/Strep. Incubated again for 3 days and collected the viral particles released into the supernatant. The viral titer was not determined and viral suspension was frozen at -80 ˚C until further use.

The transduction was performed similarly for MSCs and GSCs. I counted 100,000 cells and diluted in 10 µl of growth medium in a microfuge tube. I added 250 µl of viral suspension (thawed on ice prior to use) onto the cells into microfuge tube and incubated them for 1 hour at 37 ˚C. After incubation, I took all the mixture and added it into a single well of a 24-well plate with 1 ml of appropriate growth medium in total. For the next 3 days, I exchanged the medium with fresh growth medium, and plated the cells in a cell culture flask with a larger growth are when required. The knockdown vectors contained a puromycin resistance gene. On the 5th day, I started selected cells with appropriate doses of puromycin, determined separately for each cell type previously.

For the lentiviral transductions with a known virus titer (flipped-floxed GFP for GSCs and DsRed for MSCs), MOI of 80 was used.

3.10. RNA Isolation, cDNA Synthesis and Quantitative Real Time PCR

I isolated the total RNA from 2-5 million cells each time, using RNeasy Mini Kit (Qiagen, Cat #: 74106), which is based on binding of RNA to a resin and elution via neutralization of binding charges on the resin. After isolation of total RNA, the concentration was measured using a spectrophotometer and 1 µg was used for cDNA synthesis.

For cDNA synthesis I used QuantiTect Reverse Transcription kit (Qiagen, Cat #: 205310). This protocol involved a DNAse treatment. The cDNA mix was then used for qPCR analysis.

For the quantitative RT-PCR, I used TaqMan system from Thermo Fischer Scientific. I used the quantitative RT-PCR in order to determine the amount of knockdown and overexpression for the GSCs and MSCs I manipulated. The device I used was Applied Biosystems StepOne™ Real-Time PCR System. Negative controls (RNase- and DNase-free H_2O) were always included; GAPDH was used as positive control and as reference gene; 3 replicates were used for each condition (For detailed description, see table below).

Quantitative RT-PCR Reaction Mix Component	Volume per 20-µl Reaction (in µl)
20✕ TaqMan® Gene Expression Assay	1
2✕ TaqMan® Gene Expression Master Mix	10
cDNA template	2 µg
RNase- and DNase-free water	7

Table 1: The components of the RT-PCR reaction mix and volumes for each component per 1 reaction. The total volume was 20 µl.

The PCR protocol was applied according to the manufacturer's guidelines. The cycling settings are as follows

50 ˚C 2:00 mins

95 ˚C 10:00 mins

95 ˚C 0:15 sec ⎫ (40 cycles)

60 1:00 min ⎭

After the reaction, the fold chance in mRNA abundance for genes of interest were calculated using ΔΔCt.

3.11. Migration Assays

I cultivated a single spheroid of GBM in a single well of a 96-well plate either in MSC-CM or DMEM. Then I captured microscopic images of each spheroid at 0h, 24h, 48h and 72h using 10X or 5X objectives. I used ImageJ to calculate the area that the cells occupied in each well. For this, I determined the scaling of each image using the scale bar I added to each. I drew a line at the borders of the area cells occupied and measured the area within the closed shaped line I drew. Then, I divided the measured area by the initial area that the spheroids occupied on day 0 (determined right after plating) in order to calculate the fold change in the occupied area, and plotted the graphs based on the ratios.

3.12. Co-culture / Reporter Experiments

GSCs were transduced with a lentiviral vector (using lentiviral particles with MOI of 80) expressing a floxed- and flipped-GFP sequence. I then grew those cells and performed a single-cell colony assay. Briefly, I dissociated and counted cells, diluted the cells in a way that 1 cell will be in 300 µl of growth medium, and plated 100 µl of the mixture in each well of a 96-well plate. After the cells grew for 2 weeks, I expanded the cells. Later I divided the cell culture into two and assayed one half with the Cre-recombinase. For this, I used tat-Cre recombinase mix (Merck Millipore, Cat #: SCR508). I first titrated the tat-Cre recombinase mix for different concentrations, as it is toxic to the cells at high concentrations. I used 2 µM of the tat-Cre (Cre recombinase fused to tat protein of HIV to ensure the transport of Cre to the nucleus of the target cell as tat protein has a nuclear localization signal) mix in 500 µl of cell culture medium,

added this mixture onto 50,000 cells (in tubes) and incubated the cells at 37 ˚C for 40 mins. Then I washed out the tat-Cre mix with 10 ml of cell culture medium and plated cells into fresh cell culture flasks. After 3 days, I checked the cells under fluorescence microscope for the existence of GFP positive cells. I picked up the well containing the brightest GFP signal after tat-Cre treatment as well as containing no GFP without tat-Cre treatment, for further experiments.

Additionally, I transfected MSCs with a pCDNA-Cre-ERT2-Puro (Kindly gifted by Dr. Jennifer Altomonte) expressing a fusion gene of Cre-recombinase and ERT2 estrogen receptor. And selected the Cre-positive MSCs with application of puromycin.

I mixed those GBMs with flipped-floxed GFP with Cre-expressing MSCs at 1:1 ratio. Then I plated these cells in 20% FCS medium together and after 24h added 1ug/ml tamoxifen to the culture. 72h later, I checked for presence of GFP under a fluorescence microscope. The microscope used was Zeiss Axio Vert.A1 Inverted Microscope powered with a Mercury lamp and appropriate filters.

3.13. In Vivo Experiments

3.13.1. Injected Cells and Mice Models

I performed two different in vivo experiments. First, I co-inoculated MSCs with mouse GBM cells. For this I used a mixture of 10,000 p53KO-PDGFB-GFP cells and 10,000 mouse MSCs labeled with DsRed in 1 µl of total volume. These cells were inoculated in C57B/L6 mice brains. Later I inoculated TK-expressing GSCs. 25,000 in 1 µl of total volume of GL261-TK-eGFP cells were inoculated in C57B/L6 mice. 50,000 in 1 µl of total volume of GBM13-TK-eGFP cells were inoculated in Athymic Nude Mice (NU(NCr)-Foxn1^{nu})

3.13.2. Anesthesia

Mice were anesthesized by intraperitoneal injection of 0.1 % xylazyne and 1.5 % ketamine hydrochloride mixture in 0.9 % NaCl. The injection was performed as 10 µl of this anesthetic mixture per gram of mouse body weight. The eyes of the mice were carefully covered with glycerin cream to avoid drying of the cornea.

3.13.3. Tumor Inoculation

The tumor implantation was carried out as follows. Anesthetized mice were immobilized and mounted on a stereotactic head holder, the top of the skull (bregma) facing upwards. The skin of the top of the head was incised and the skull surface was disinfected. 1 mm anterior and 1,5 mm lateral to the bregma, the skull was pierced with a 20-gauge needle. Then a 1 µl microsyringe was inserted till a depth of 4 mm and retracted to 3 mm, to make space and the cell suspension was injected into that space. The needle was slowly taken out and the skin was sutured. An antibiotic cream was applied on the sutured region.

3.13.4. Ganciclovir Administration

In order to treat mice with GCV, 2 strategies were followed. In the first approach, the GCV was administered systematically via intraperitoneal injection of a dose of 25 mg/Kg body

weight/animal/day. In the other approach, osmotic mini pumps were used. Those pumps were loaded with µl 200 of 2 mM GCV solution. After tumor growth of 7 days, both treatment regimens were applied for the next 7 days or 14 days.

3.13.5. Perfusion and Brain Fixation

For fixation, the mice were anesthesized and perfused with an intracardiac infusion of 0.9% NaCl solution, followed by ice cold 4% paraformaldehyde (PFA) solution. Later, the skull was broken gently and opened and the brain was postfixed overnight in 4% PFA. Finally, the brains were maintained in 30% sucrose solution for cryopreservation.

The staining for mice brain inoculated with TK-expressing GSCs was performed with Hematoxylin and Eosin staining.

3.14. Data Analysis

All the data analysis was performed using student's non-parametric, un-paired t-test. For analysis of data, Microsoft Excel was used and for plotting the graphs and performing statistical analyses, Graphpad-Prism was used. Significances were depicted as *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. Data were presented as mean ± St.dev.

4. RESULTS

4.1. CM from MSCs Modulate the Viability of GBMs *in vitro*

CM was produced from bone marrow-isolated MSCs that were cultivated in either with serum or serum-free conditions for 72 hours and the viability of an MSC-stimulated GBM cells called GBM20 was assessed which are primary GBM cells with stem-like characteristics. The GBM viability is negatively affected by the CM from MSCs cultivated under serum-containing conditions. Interestingly, the GBM viability was enhanced when they were stimulated with CM from MSCs kept under serum-free conditions. These effects were observed with MSC from human bone marrow (BM-6, BM-30) or MSC purified from human GBM (gb863; Figure 1).

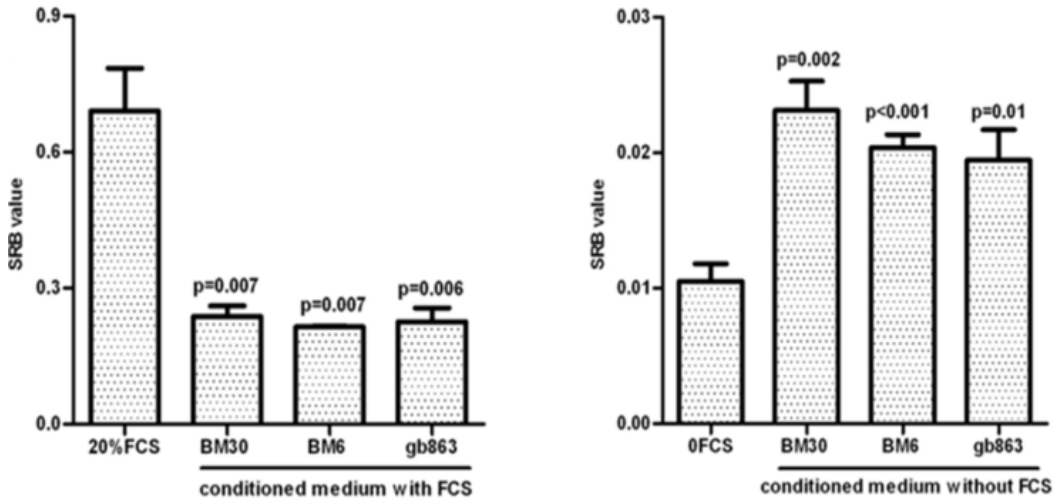


Figure 1: Presence or absence of serum determines the pro- or anti-tumorigenic effects of MSCs **Left Panel:** CM from MSCs (from BM 30, BM6 or gb863 MSCs) that were maintained in the presence of serum for 72h was applied on GBM20. After incubation for 72h, the viability of GBM20 was drastically reduced. **Right Panel:** CM from MSCs cultivated in serum-free conditions for 72 hours strongly increased the viability of GBM20. (Compared to unconditioned medium – 20% FCS and 0 FCS, respectively). The data were analyzed using Nalimov test. Controls were arbitrarily set as 1. The differences as compared to controls are statistically significant ($p < 0.005$).

4.2. MSCs Home Satellite Tumors in vivo

I then wanted to investigate, which of these two in vitro conditions is more relevant to the pathological situation where MSCs settle in brain tumors in vivo (i.e. if MSCs are in contact with blood-serum or they are clustered in areas where blood-serum cannot penetrate). I orthotopically inoculated transgenic mouse glioma cells (p53KO-PDGFB-GFP) into the brains of immunocompetent mice and 10 days after tumor inoculation, injected genetically labeled

MSCs (expressing red-fluorescent protein; RFP). The brains were dissected 20 days after tumor induction. As an alternative approach, I co-inoculated mouse glioma cells together with RFP-expressing MSCs (not shown). In both experimental situations, I observed that, in the main tumor mass, there are very few MSCs while in the invasive front of the glioma (satellite tumors located distantly from the main tumor mass) the MSC:GBM cell ratio was 1:1 (Figure 2).

After this observation, I asked the question: why do we see only very few MSCs in the main tumor mass despite the relatively high number of MSCs in the satellite tumors? So, I repeated the experiment with the same cells in a sequential manner (i.e. MSCs were injected after tumor induction). This time I waited only 3 days after MSC injection and dissected the brains. Upon this new setting, I was able to observe relatively larger amounts of MSCs in the periphery and center of the established gliomas. From this observation I concluded that, MSCs have a relatively short life span in the main tumor mass and probably disappear within a few days. Yet, MSCs seem to persist for longer periods of time in the satellites, probably, due to absence of ever-changing pathological situations and presence of more stable conditions.

From the above-mentioned in vivo experiments, I concluded that MSC-GBM interaction is more stable and relevant in the invasive tumor parts which are buried deep in the tumor parenchyma and often located distantly from the main tumor mass. These invasive tumor parts are devoid of histopathological hallmarks of glioma, such as hypoxia, inflammation, bleedings or necrosis, which, therefore, is a major obstacle in diagnosis and treatment of GBM (Jermyn et al., 2016). Given these circumstances, I hypothesized that, MSC-GBM cell interaction in invasive tumor front is more relevant and it is best recapitulated in vitro by serum-free conditions as blood-derived factors cannot reach such structures that lack bleedings.

The tumor promotion by the CM from MSCs cultivated in serum-free conditions was demonstrated previously for GBM20 (Figure 1). Next, I wanted to evaluate how consistent this effect of MSCs is observed for different GSCs. Upon stimulation, CM from MSCs cultivated in serum-free conditions enhanced the viability of a range of GSCs. The increase in viability varied drastically among different GSCs ranging from 1.5X to 10X (Figure 3). Proneural subtype GBMs (GBM10; GBM13; Line 7; Line 9) displayed a larger increase in viability than classical and mesenchymal subtypes. Due to this heterogeneity in response, GSCs with higher levels of viability increase, Line 4, Line 7 and GBM13 were selected for further experiments.

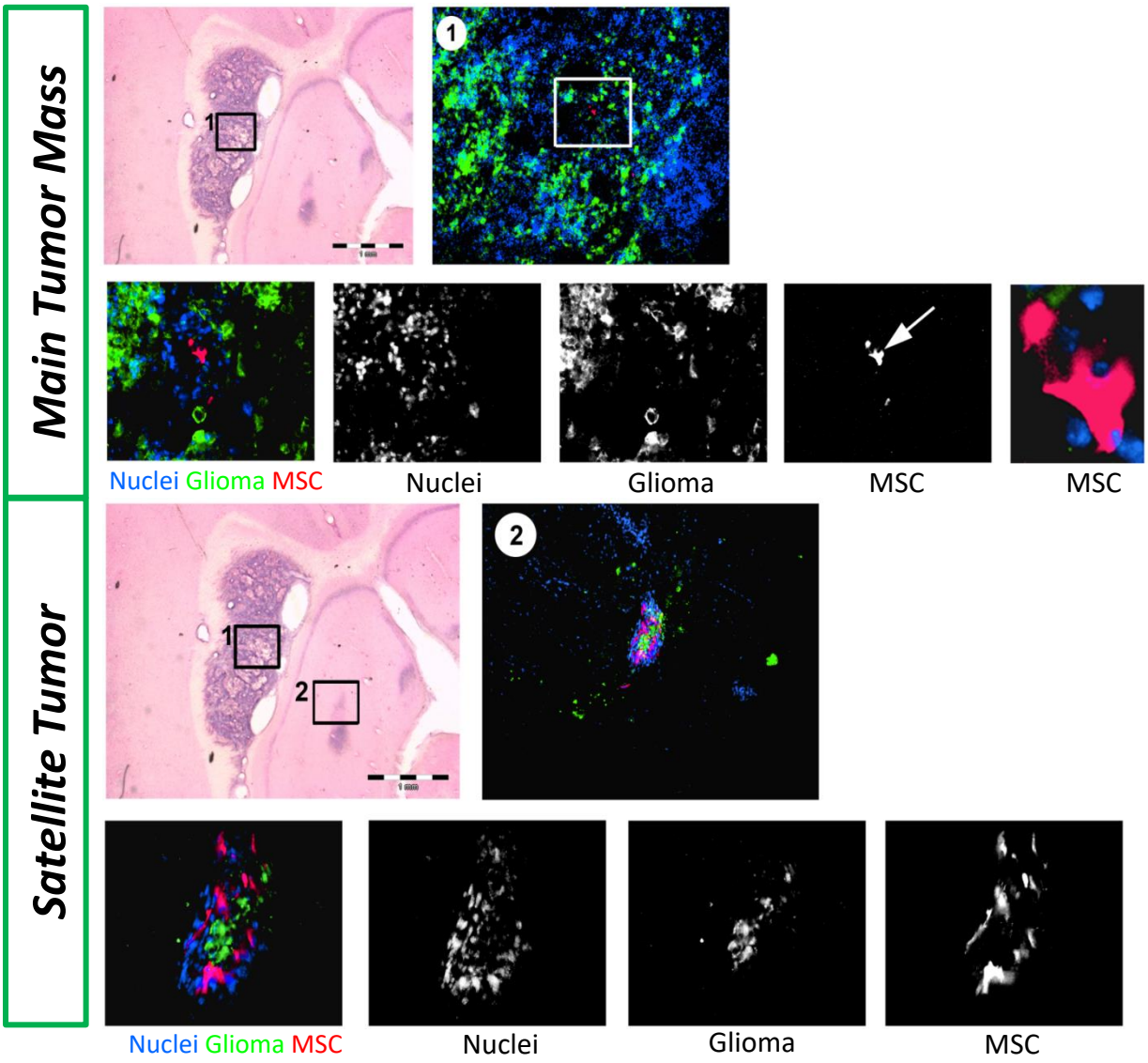


Figure 2: MSCs migrate and integrate into the tumor satellites. Mouse glioma cells (p53KO-PDGFB-GFP) were inoculated together with RFP-expressing MSCs. GBM cells proliferated and formed a large tumor mass (Indicated as "Main Tumor Mass") along with small satellites (Indicated as "Satellite Tumor"), shown with Hematoxylin-Eosin stainings in left panels of 1st and 3rd row, respectively, where square numbered as 1 shows the main tumor mass and square 2 shows satellite. MSC numbers in main tumor mass are significantly low, depicted in 1st row – right panel as well as by arrow in the second row where blue color indicates nuclear staining, green indicates tumor cells and red shows MSCs. The 2nd row shows a close caption of the area indicated in square 1. On the contrary, MSCs are more numerous in invading glioma parts than in main tumor mass (shown in "Satellite tumor", square 2). MSC:GBM cell ratio is nearly 1:1. Individual cell populations from a single tumor satellite are shown in single channel views, in 2nd and 4th rows for main tumor mass and satellite tumor, respectively. Scale bar is 1mm.

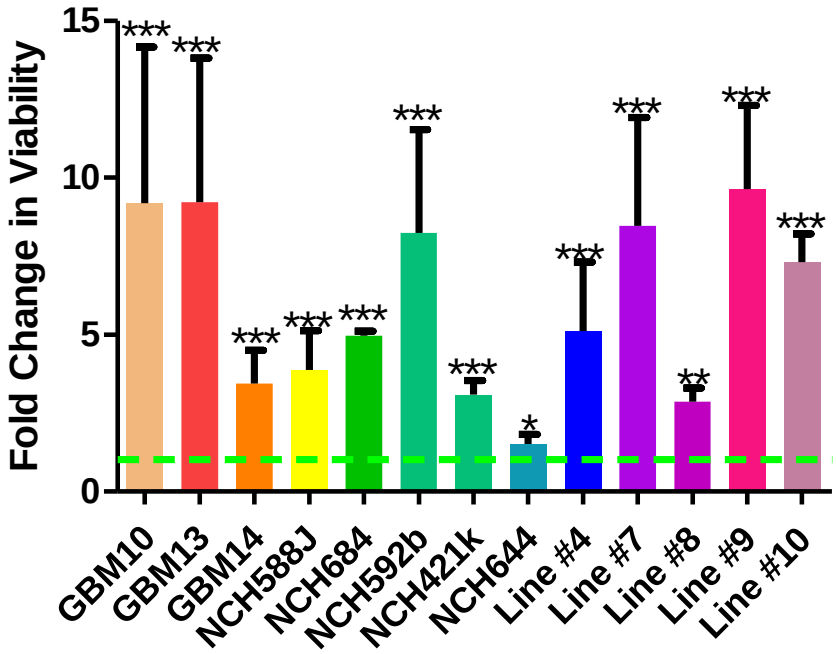


Figure 3: MSC-CM (serum-free) increases the viability of GSCs *in vitro*. GSCs were subjected to 72h of stimulation with CM from MSCs cultivated in serum-free conditions. The fold change in viability was described as normalized to the control (non-conditioned plain cell-culture medium) using MTT assays. Data are expressed as fold-change from control levels which were arbitrarily set as 1 (indicated with the dashed green line). Statistical significance is shown as: *** $p < 0.001$, ** $p < 0.005$, * $p < 0.05$.

4.3. MSC-CM Induces the Expression of QKI in GBMs

My next direction of interest was the molecular signaling mechanism of CM-induced viability increase. I analyzed the differentially regulated genes in CM-stimulated GBM13, a GSC that responds to MSC-CM by a massive increase in cell number and viability (Figure 3). I exposed GBM13 to MSC-CM (serum-free) for 24h, 48h and 72h or DMEM (control medium), harvested cells and performed a gene expression analysis via microarray (Figure 4). We used a biclustering approach in order to avoid the "noise" caused by large set of genes due to massive increase in cell number. Biclustering analysis allows for detection of networks of genes and can reveal genetic nodes controlling large physiological pathways [Cheng and Church, 2000]. In biclustering method, both genes and samples are compared simultaneously. Comparing samples and gene expressions via a biclustering approach in expression matrix, setting significance $p \geq 0.05$, we diminished the number of target pathways. This way, the clustering of genes and samples (also according references in the KEGG-database) converged onto pathways which had a specific gene called Quaking (QKI) at the central nodule, shifting further away from the controls in a time-dependent manner, showing significance and constant increase in expression. Therefore, I concluded that QKI is significantly associated with survival and proliferation.

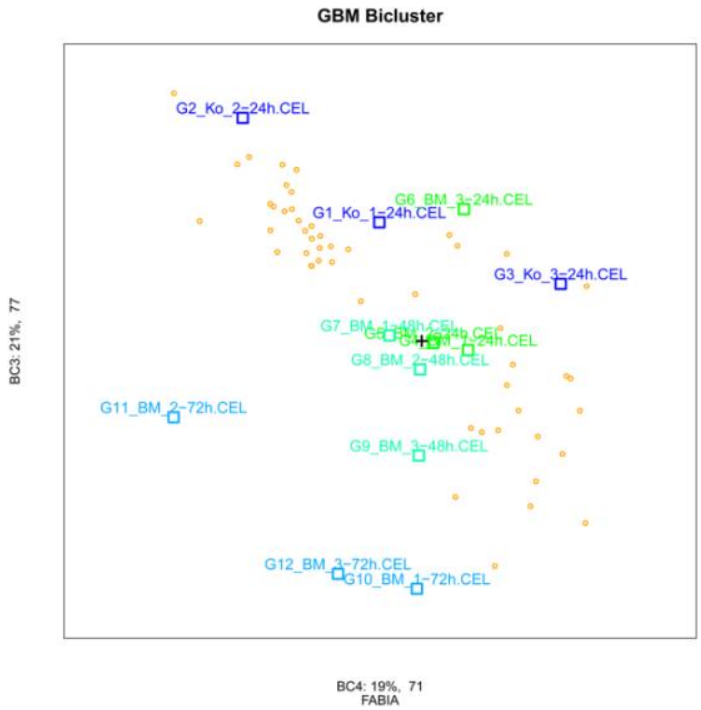


Figure 4: QKI emerged as a candidate from bioinformatics-analysis of coregulated genes. GBMs were treated with CM for 24, 48 and 72 hours. The pellets were collected and microarray analysis was performed. Global changes in gene expression patterns are graphically indicated. Initially controls (dark-blue) and GBM-cells treated for 24h (green) are relatively close, the treated cells shift away from controls after 48h (turquoise) or 72h (light-blue) of stimulation with MSC-CM. The more the treated cells are in close proximity to the controls on this plot, the less significant the change in expression of a certain gene is. The pathways that are activated with an increase in activity in a time dependent manner were identified. QKI emerged at the central nodule in these pathways.

4.4. Manipulation of QKI in GBMs and EFNA3 in MSCs

In order to define specific roles of QKI in GBMs in the above-mentioned network, I manipulated the expression levels of QKI in different GBM cells by knocking down or overexpressing it. QKI has three isoforms, namely QKI-5, QKI-6 and QKI-7. Overexpression was achieved by transfection of GBMs (GSCs named Line-4 and Line-7 due to higher proliferation rate and easier transfection or transduction than GBM13, which is to be included later on) with plasmids encoding separate isoforms (Figure 5, upper graphs). For the knockdown, however, I had to use a viral vector encoding an ShRNA which targets the core region shared by all three QKI isoforms, simultaneously instead of targeting each isoform separately. With these plasmids I was able to overexpress QKI-5 (5-fold in Line 4 and 2,5-fold in Line 7), QKI-6 (6-fold in Line 4 and 5,9-fold in Line 7), and QKI-7 (4-fold in Line 4, and 3,8-fold in Line 7) (Figure 5 upper panel). Knockdown of QKIs was achieved up to 98% (Figure 5 lower panel).

4.5. QKI promotes viability of GBM

After confirming the overexpression of QKI, I performed a viability assay with GSCs Line 4 and Line 7 that overexpress QKI isoforms QKI-5, QKI-6 and QKI-7. In these lines, overexpression of QKI isoforms lead to an increase in viability compared to those transfected with vehicle control (empty) in control medium, DMEM. QKI overexpression even further boosted the viability increase by MSC-CM (Figure 6, upper panel). In concordance with this, knockdown of QKIs lead to a significant decrease in viability compared to nontargeting vehicle control (NT) in DMEM. Knockdown could even blunt the effect of MSC-CM in Line 7 but not in Line 4 (Figure 6, lower panel). In light of these findings, I concluded that, QKI overexpression is one possible explanation for the increase in GBM viability when they are cultivated in MSC-CM. However, more cell lines need to be assessed in the same way. Moreover, subjection of QKI knockout GBMs to CM from MSCs (serum-free) might shed more light onto this phenomenon. Moreover, mass spectrometry of the GBMs stimulated with the MSC-CM might help identification of specific molecules.

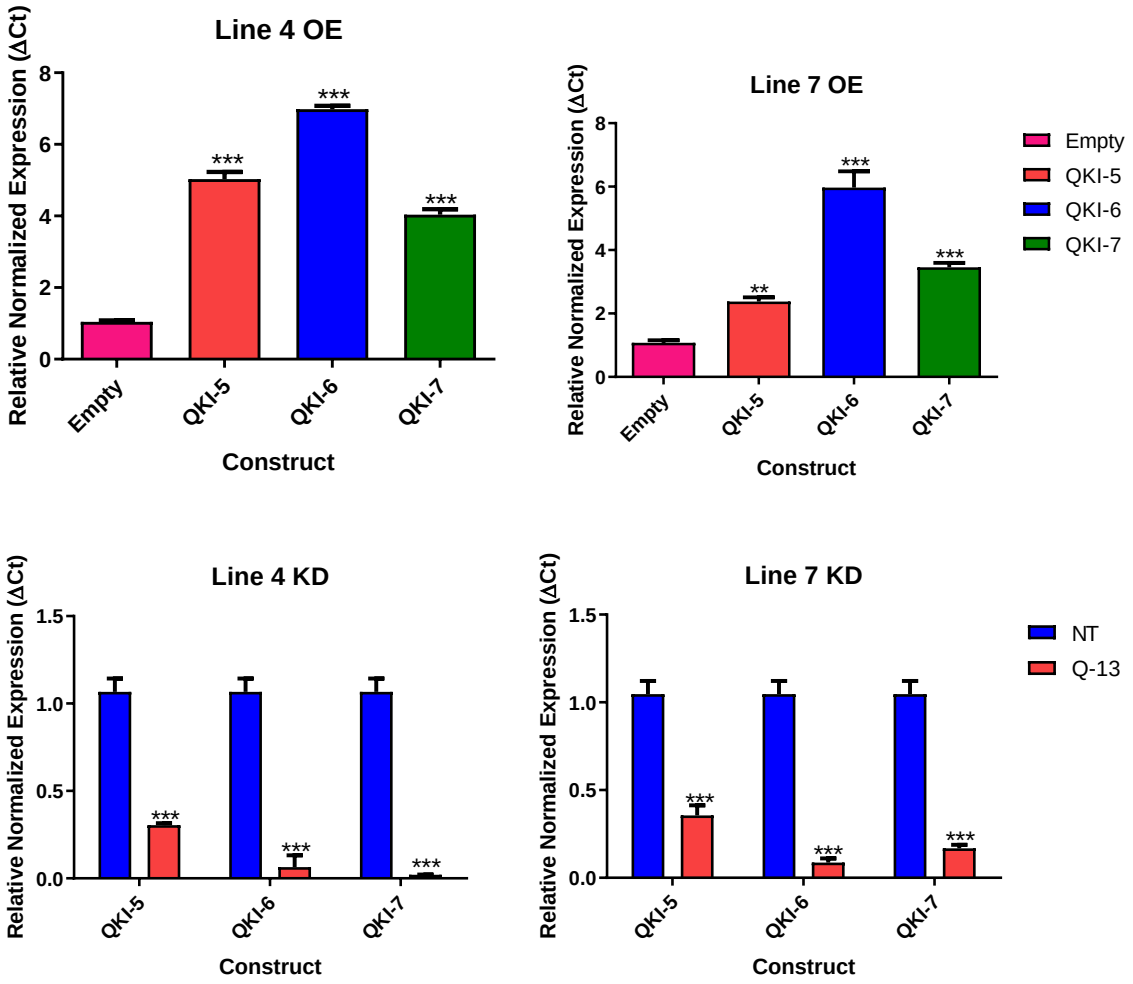


Figure 5: Overexpression and Knockdown of QKI. QKI isomers were overexpressed using specific expression vectors for each isomer (Upper Panel). For line 4, this overexpression was 4,8-fold for QKI-5; 6,9-fold for QKI-6 and 3,9- fold for QKI-7 (Upper Left Panel). For Line 7, it was 2,7-fold for QKI-5; 6,1-fold for QKI-6 and 3,8-fold for QKI-7 (Upper Right Panel). For Line 4, the knockdown yielded 62% decrease for QKI-5; 90% decrease for QKI-6 and 98% fo QKI-7 (Lower Left Panel). For Line 7, the decrease for QKI-5 was 60%; 95% for QKI-6 and 68% for QKI-7 (Lower Right Panel). The relative expression of QKI isoforms in both settings are indicated as means normalized to the amount of endogenous GAPDH mRNA for each sample ± SD from triplicate data. Normalization was carried out by ΔΔCt method. Statistical significance is shown *** $p < 0.001$, ** $p < 0.005$

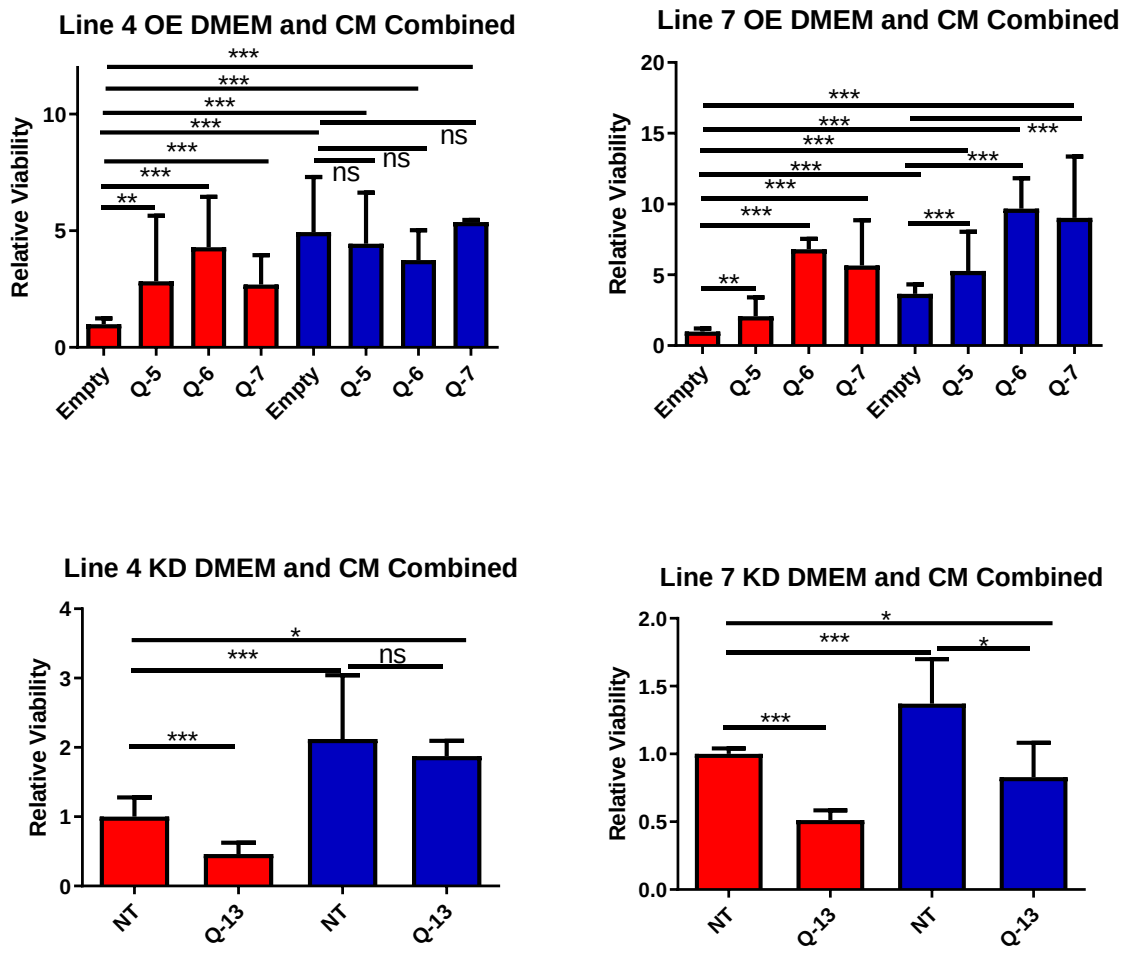
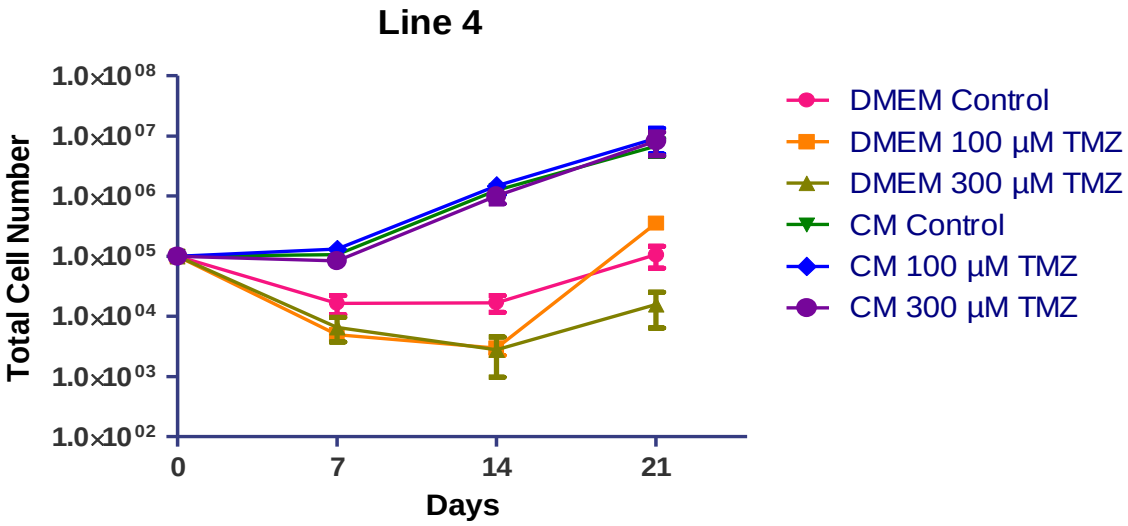


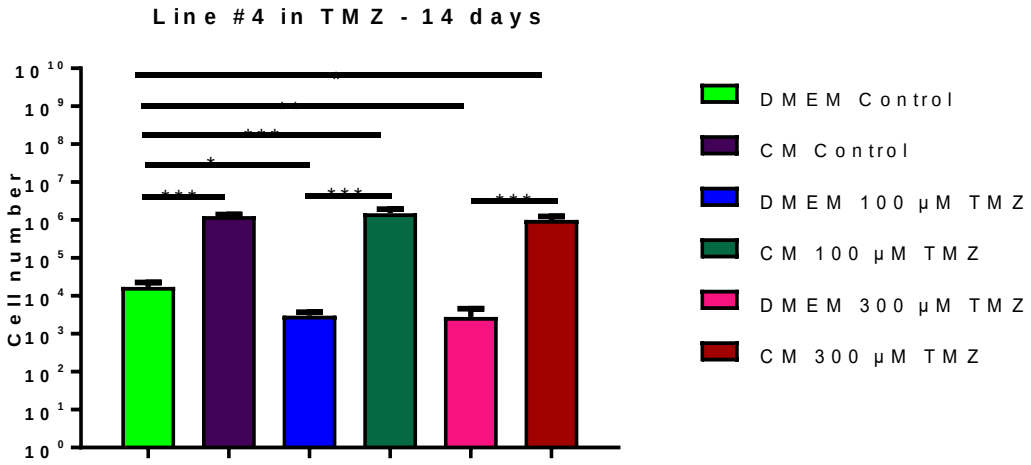
Figure 6: QKI-overexpression and -knockdown in GBMs modulate viability and MSC-CM induced effects. **Upper panel:** QKI-overexpression in Line 4 increases the viability in basal conditions (DMEM) up to 2,8-fold for QKI-5 and -7 and 4,7-fold for QKI-6 compared to vehicle control (Empty). However, this overexpression does not yield a further increase in CM, compared to vehicle control that is cultivated in CM, too. For Line 7, QKI-5 overexpression yielded 2,3-fold increase in viability, whereas, QKI-6 caused a 6,9-fold, and QKI-7 caused a 6,4-fold increase in basal conditions (in DMEM). The overexpression of QKIs, however, yielded a further increase in viability in CM, compared to vehicle control that is also incubated in CM. **Lower panel:** In line with this, knockdown of QKIs lead to a decrease in viability in DMEM, up to 52% for Line 4 and 50% for Line 7. When incubated in CM, this knockdown did not cause any decrease for Line 4, however, for Line 7, it completely blunted the effect of MSC-CM. Red bars indicate Control Medium (DMEM) and Blue bars indicate CM. Statistical significance is shown *** $p < 0.001$, ** $p < 0.005$, * $p < 0.05$

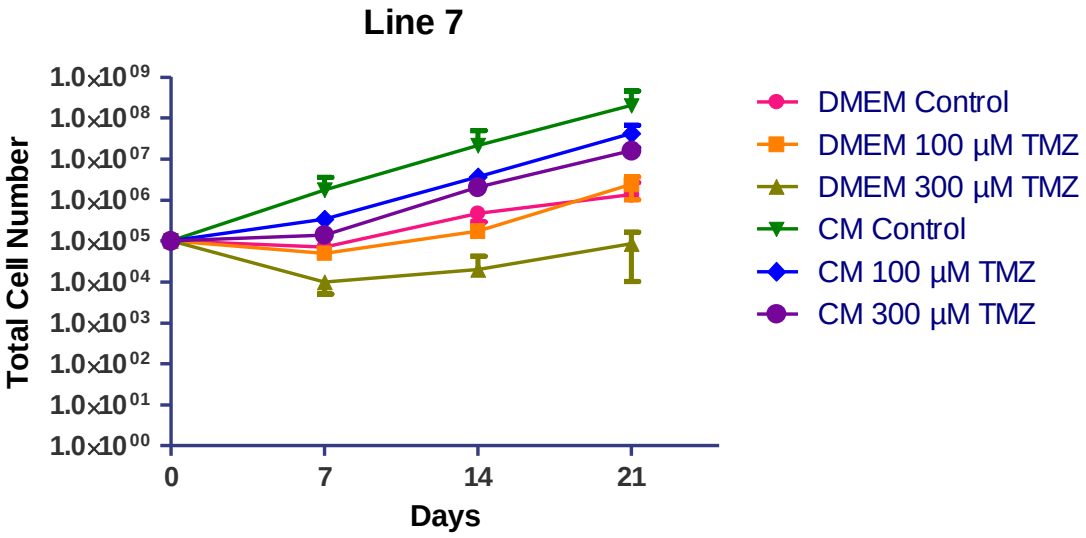
4.6. MSC-CM mediates chemoresistance of GBMs

So far, I have demonstrated that MSC-GBM interaction mainly takes place in the invasive front (i.e. tumor satellites – Figure 2) in vivo. The MSCs migrate to tumor satellites and settle more than the main tumor mass. The interaction of MSCs with GBMs leads to an enhanced tumor cell viability in vitro although the increase in viability in vitro is heterogeneous among different GBM cell lines I used (Figure 3). My aim was to determine if this situation is clinically relevant in terms of therapy. During the treatment of a brain tumor with conventional ways, the main tumor mass is resected which is followed by a chemo- and radio-therapy. This method of treatment, however, cannot eliminate the satellite tumors, which poses a significant risk of tumor relapse. As the MSCs improve the GBM cell viability in vitro under serum-free conditions, which corresponds to the tumor satellite vicinity lacking blood-borne molecules (i.e. MSCs mainly migrate towards satellite tumors), they potentially act in the same way in vivo as well. Therefore, there is an eminent need of averting the MSC-mediated tumor cell survival. That is why I also asked if MSCs help tumor cells parry the effects of chemotherapeutic agent temozolomide (TMZ), which is the conventional chemotherapy drug used to treat GBMs. Therefore, I tested the sensitivity of our GSCs to TMZ and observed that the extent of sensitivity to TMZ varies among GSCs, monitored by the extent of cell death (data not shown). Next, I applied TMZ in combination with MSC-CM onto GBMs that were sensitive (Figure 7 – upper panel, Line 4) and slightly resistant (Figure 7 – lower panel, Line 7) to TMZ. In this experiment, I applied TMZ as pulses by refreshing the medium containing TMZ every 24h for 72h and monitored the long-term survival of GSCs in vitro, in order to mimic the clinical situation where pulses of TMZ are given to the patients. Additionally, TMZ is an alkylating agent whose effects on cell proliferation and survival manifest itself in the long term. Consequently, I found out that, MSC-CM largely increases GBM cell numbers (Figure 7) compared to relevant controls (i.e. TMZ-treatment in control medium; DMEM; please note that changes in cell-numbers are quite large after MSC-CM application, data are given on a log-scale). The rescue of GBMs from TMZ-induced cell death by MSC-CM was best visible on day 14 of the incubation (Figure 7, bar graphs on the right of both upper and lower panels). This is because, after the pulse application of TMZ, GBM cells start to recover, and these cells in addition to unaffected or less-effected cell population takes over the cell culture after this time point.

All in all, I demonstrated that MSC-CM massively induces chemoresistance in GBM cells. Differences in cells treated with 100 µM or 300 µM TMZ alone or TMZ together with MSC-CM accounted to 4 orders of magnitude after 21 days of investigation. The effect of TMZ, however, is heterogeneous and the rescue effect of MSC-CM varies among different GBM cell lines. Moreover, the GBMs that received TMZ in MSC-CM had comparable cell numbers to those received only MSC-CM (control) showing how potent the MSC-CM in negating the effects of TMZ in terms of cell death. The rescue effect of MSC-CM from TMZ-induced cell death was not only limited to those two GSC lines (Line 4 and Line 7) but also prominent in many more of the GSC lines we have (Supplementary Figure 1).


Line 4
Total Cell Number
1.0×10^08
1.0×10^07
1.0×10^06
1.0×10^05
1.0×10^04
1.0×10^03
1.0×10^02
0
7
14
21
Days
DMEM Control
DMEM 100 µM TMZ
DMEM 300 µM TMZ
CM Control
CM 100 µM TMZ
CM 300 µM TMZ


Line #4 in TMZ - 14 days
Cell number
DMEM Control
CM Control
DMEM 100 µM TMZ
CM 100 µM TMZ
DMEM 300 µM TMZ
CM 300 µM TMZ



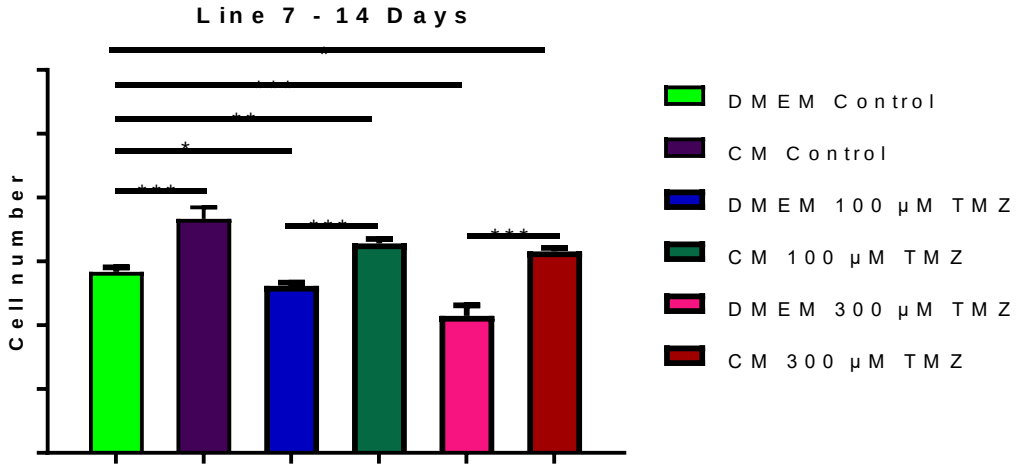


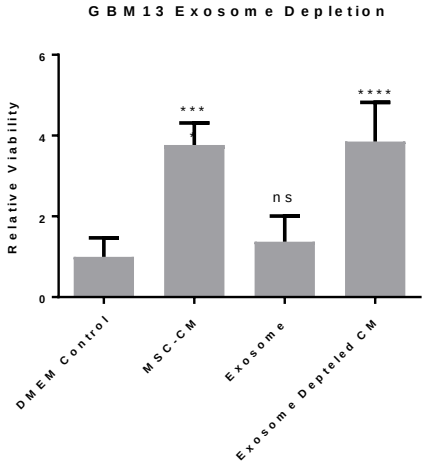
Figure 7: MSC-CM renders GSC lines 4 and 7 chemoresistant. I applied TMZ repeatedly for 72h, with 24h intervals on the first 3 days as pulses by refreshing the medium containing TMZ. On days 7, 14, 21, I counted the cells and used 3 replicates for each condition (for detailed methodology, see methods – Chemoresistance Assay). The cell numbers were determined, then 100,000 cells were plated back. The final numbers on each count (14 and 21 days) were calculated assuming all the cells were plated back using the equation 100,000xA = BxC where A=counted cell number (on day 14 or 21), B=total cell number from previous count (on day 7 or 14), C=total cell number if all cells were plated (on day 14 or 21). The total cell numbers (corresponding to B on day 7; C on day 14 and 21) were plotted on a semi-log scale (Y-axis as logarithmic scale, X-axis as normal scale – days) for scatter-plots and for the bar graphs (demonstrating the largest difference in cell numbers which was observed on day 14). Data are presented as Mean ± SD. Statistical significance is shown *** $p < 0.001$, ** $p < 0.005$, * $p < 0.05$

4.7. MSC-released Soluble Factors largely account for Increased GBM Viability and Chemoresistance

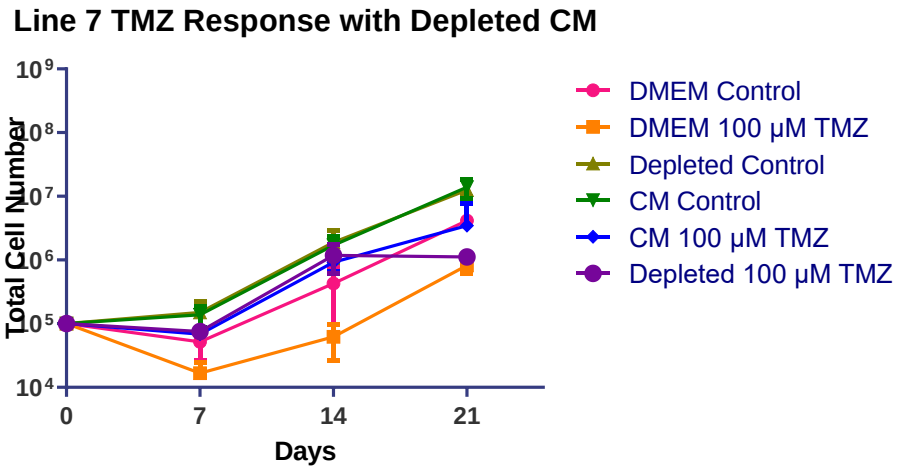
It was shown by others (Baglio et al., 2012) and us that many pathologically important effects of MSCs are induced via MSC-released extracellular microvesicles called exosomes. So, I asked if the viability increase and chemoresistance are mediated by exosomes or soluble factors.

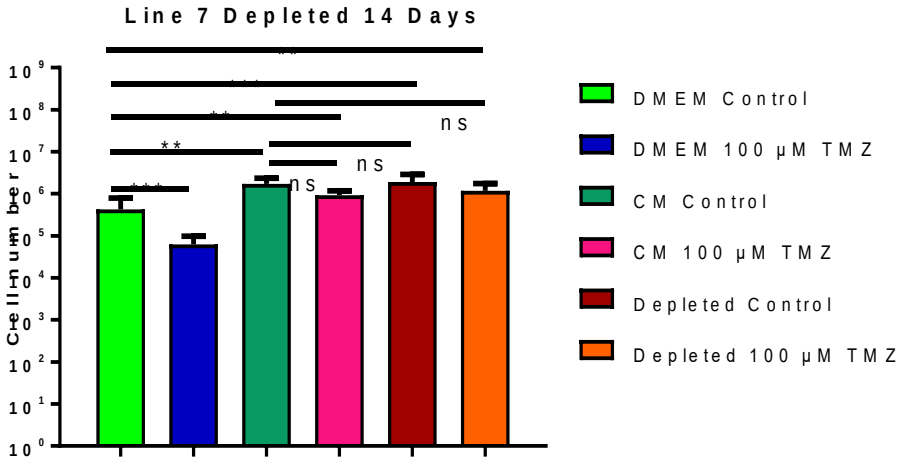
To answer this question, I performed two different experiments. First, I depleted exosomes from CM as well as isolated exosomes from CM and applied onto GBM13 as the microarray data were obtained using GBM13 originally, and GBM13 was one of the GBM lines which gain the largest increase in viability upon CM application. Exosomes failed to induce an increase in the viability, however, exosome depleted MSC-CM mediated an increase in the viability, at a comparable level to that of MSC-CM (Figure 8- a). In the second phase, I applied the TMZ with the same settings described above and used either exosome-containing or exosome-free media. I observed that both exosome-containing and exosome-free media from MSC-CM resulted in significantly more numerous cell counts compared to control DMEM over 21 days after TMZ treatment (Figure 8-b). This situation can be explained by the biochemistry of the signaling molecules conferring the observed effects. It was previously shown by Roodhart et al. that MSCs mediate chemoresistance by releasing bioactive fatty acids (Roodhart et al., 2011). The bioactive lipid that mediates the increase in cell viability in the presence of TMZ treatment accumulates in the lipid phase of the CM along with the exosomes, which is also rich in membrane-related lipids. On the other hand, I noted that the exosome fraction is not necessary for MSC-CM to confer chemoresistance (exosome-free CM also induces chemoresistance of GBMs). In conclusion, a soluble factor (likely a bioactive lipid as MSCs have been shown to induce chemoresistance in cancer cells by Roodhart et al. [Roodhart et al., 2011]) is responsible for the induction of the observed increased viability and chemoresistance in GBMs.

A


GBM13 Exosome Depletion
Relative Viability
0
2
4
6
***
ns
****
DMEM Control
MSC-CM
Exosome
Exosome Depleted CM

B


Line 7 TMZ Response with Depleted CM
Total Cell Number
10^9
10^8
10^7
10^6
10^5
10^4
0
7
14
21
Days
DMEM Control
DMEM 100 µM TMZ
Depleted Control
CM Control
CM 100 µM TMZ
Depleted 100 µM TMZ


Line 7 Depleted 14 Days
Cell number
ns
ns
ns
DMEM Control
DMEM 100 µM TMZ
CM Control
CM 100 µM TMZ
Depleted Control
Depleted 100 µM TMZ

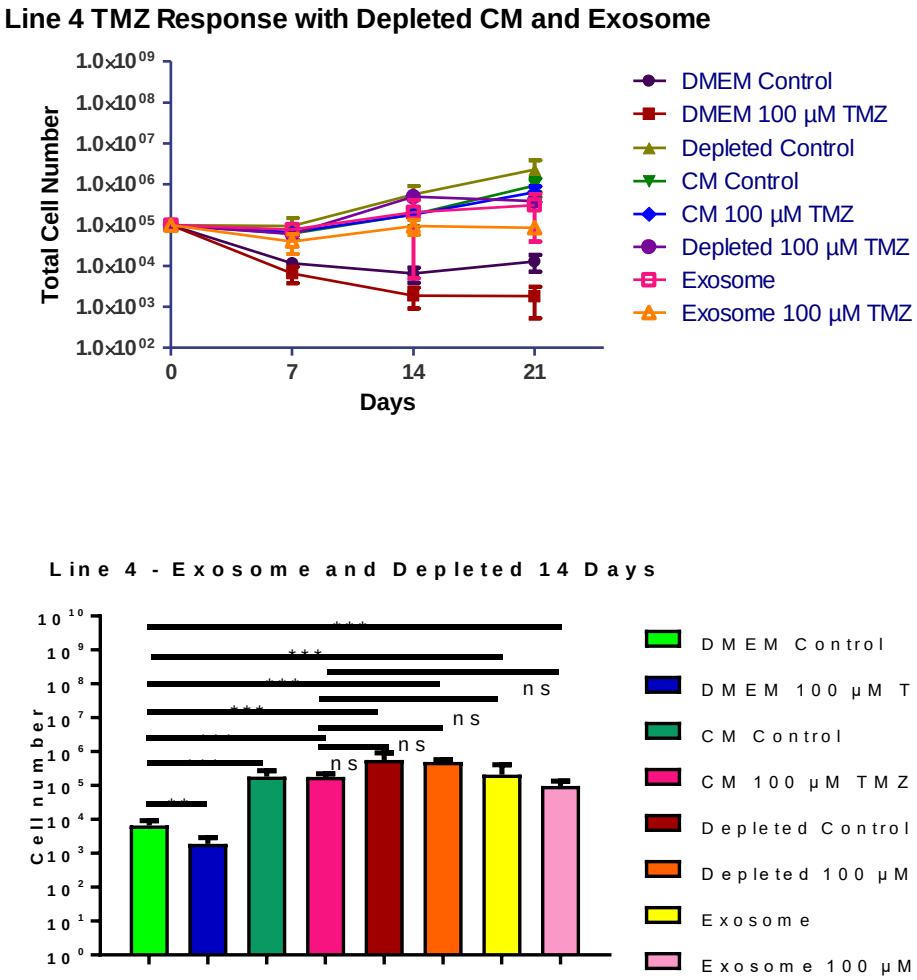


Figure 8: MSC-released soluble factors confer viability and chemoresistance. **A.** Exosomes were depleted by ultracentrifugation or isolated from MSC-CM. They were then applied onto a GSC line, GBM13. The exosomes failed to boost the viability of GBM13, however, exosome depleted MSC-CM induced an increase in viability, at a comparable level to that of MSC-CM alone. **B.** GSCs were challenged with TMZ in the presence of conditioned media from MSCs (CM), purified exosomes, exosome-free CM (depleted) or with non-conditioned media (DMEM). Exosome-depleted medium was fully capable of mediating TMZ resistance in GBM cells (Depleted) The observed cell numbers were comparable to those of GSCs subjected to TMZ in the presence of CM. The cell counts when GSCs were maintained with exosomes were not statistically different from controls, showing that exosomes do not confer a chemoresistance, as shown for Line 4 (Lower Panels). The rescue effects of CM and Depleted media were most pronounced on day 14 after TMZ treatment (Left Panels, Scatter Plots. GBM Cells start to recover after that time point – See Chemoresistance Assay), and cell numbers on day 14 were shown by bar graphs (Right Panels on a log-scale). Data are indicated as Mean ± SD. Statistical significance is shown *** $p < 0.001$, ** $p < 0.005$, * $p < 0.05$

Quaking-6 (QKI-6) Mediates the MSC-CM-induced Increase in Viability and Chemoresistance

Previously I have shown that the QKI expression is induced in GBMs by MSC-released soluble factors and that QKI expression is necessary for GBMs to gain an increased viability (Figure 6). Therefore, my next question was if increased QKI expression alone is capable of inducing the above-mentioned chemoresistance in GBMs. In order to investigate that scientific question, I utilized the GSCs overexpressing QKI (three different isoforms named QKI-5, QKI-6 and QKI-7) and treated them with TMZ. I demonstrated that QKI expression (for all three isoforms) alone was able to confer chemoresistance in GSCs. However, the rescue was most pronounced for QKI-6, whereas QKI-5 and QKI-7 caused a modest rescue Line 4 from the effects of TMZ (Figure 9).

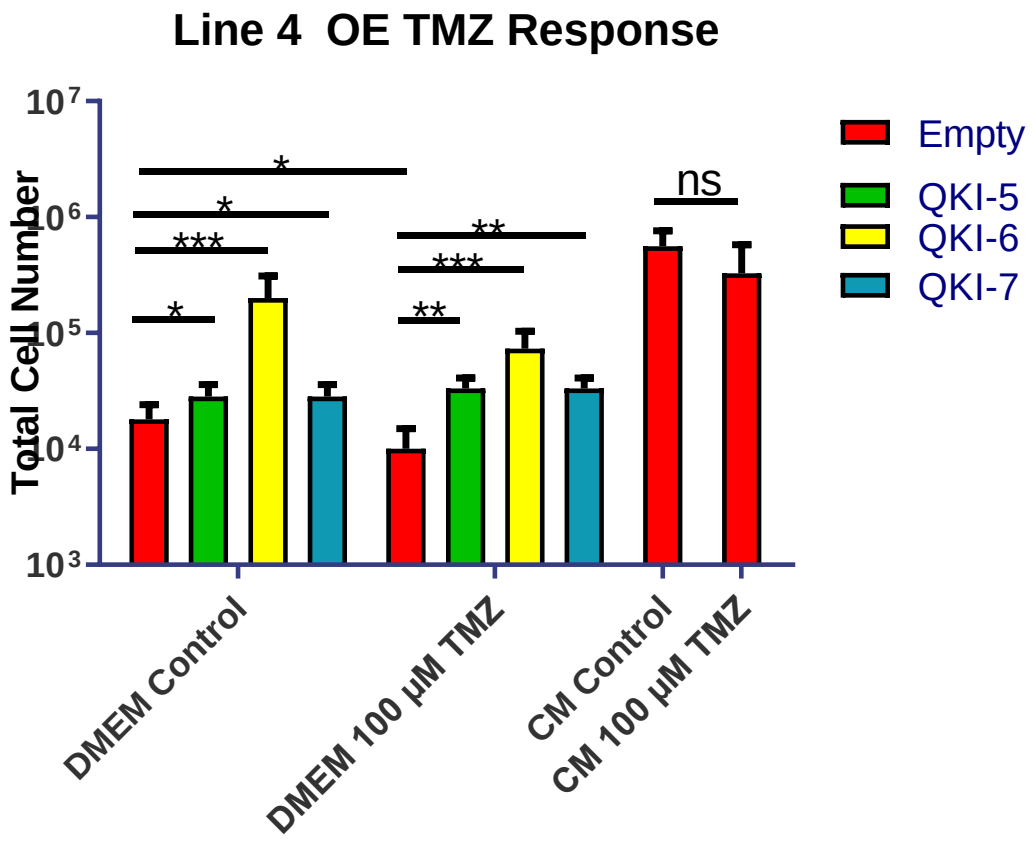


Figure 9: Overexpression of QKIs, is sufficient to induce TMZ-resistance in GBM. GSC line 4 either overexpressed different QKI-isoforms (QKI-5; QKI-6 and QKI-7) or contained an empty control vector (red bar). GSCs with empty vector were sensitive to TMZ treatment and acquired chemoresistance by MSC-CM. Overexpression of QKI (most strongly QKI-6 isoform depicted by yellow bars) was sufficient to induce chemoresistance in GSCs. Differences between the DMEM and DMEM-QKI-6 samples are statistically significant ($p < 0.005$). Data were presented as Mean ± SD.

4.8. QKI de-repress EGFR

I showed earlier that, a soluble factor (likely a bioactive lipid) is required to induce changes in the gene expression pattern in GSCs stimulated by the MSC-CM, where QKIs play an important role. Moreover, MSCs induce the chemoresistance in GBMs in vitro and QKIs are one important factor in this interplay. Additionally, knockdown of QKI expression levels caused a significant reduction in the viability of GBMs under control conditions and negated the induction of viability increase in the presence of MSC-CM stimulation (Figure 6 – Lower Panels). That is why it was important to know how QKIs have such drastic influences on GBM cell viability. Additionally, QKI is an intracellular signaling factor, which proves to be inaccessible for targeting for therapeutic purposes. With these in mind I investigated the molecular stakeholders of this signaling network and found out that, reductions in QKI levels by knockdown causes a decrease in the expression levels of EGFR (Figure 10). It was shown by others (Wang et al., 2013) that QKI, being a member of STAR – RNA binding protein family, can sequester MiRNA-7 in nucleus in the form of pri-MiRNA while being processed. MiRNA-7 targets EGFR and the abundance of QKIs increase the sequestering, thereby increasing the abundance of EGFR, which promotes cell division and survivability. Currently open question is if EGFR blockers can have beneficial effects in terms of GBM treatment, also in combination with TMZ.

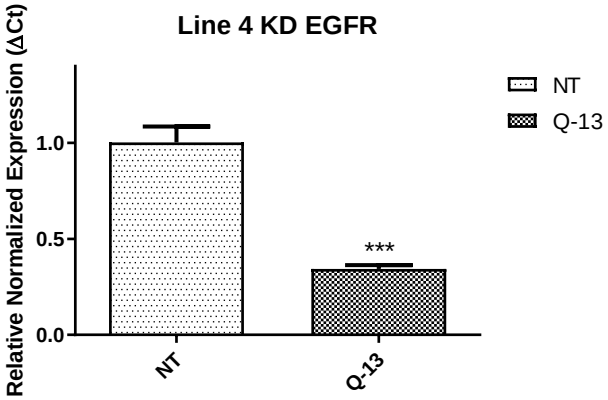


Figure 10: QKI-knockdown results in a decrease in EGFR levels in Line 4. Quantitative real-time PCR analysis of total RNA from Line 4, with QKI knockdown, using TaqMan probes directed to EGFR displayed a decrease in EGFR levels in the QKI-knockdown cells (Q-13), compared to controls (NT). The difference between control and the knockdown bar is statistically significant (*** $p < 0.001$). This shows that QKI knockdown causes a decrease in EGFR levels.

4.9. MSC-derived Exosomes Promote GBM-invasion via EFNA3 Induction

Previously I demonstrated that the interaction between GBMs and MSCs mainly takes place in the invasive areas of the tumor i.e. tumor satellites (Figure 2). Here, MSCs might be supporting the viability of GBMs while they are invading the brain, which in turn lead to a tumor relapse in the long term. GSC cultures grow as spheroids under control conditions in vitro. However, during my experiments where I measured the viability of GBMs upon serum-free MSC-CM and exosome stimulation, I noticed that MSC-CM causes an adherence followed by a morphological change in the recipient GBMs. In order to test this further, I cultivated GBMs in serum-free MSC-CM or in the presence of MSC-released exosomes dissolved in control medium. I observed that GBM cells undergo drastic morphological changes when incubated in MSC-CM and in exosome-medium, reminiscent of motile cells, which was not observed in exosome-free and control medium (Figure 11). However, those morphological changes were not observed in all GSC lines, but mostly limited to proneural and mesenchymal subtypes of GSCs. Therefore, I chose the GSC lines with relatively high levels of morphological changes for further experiments regarding exosomes.

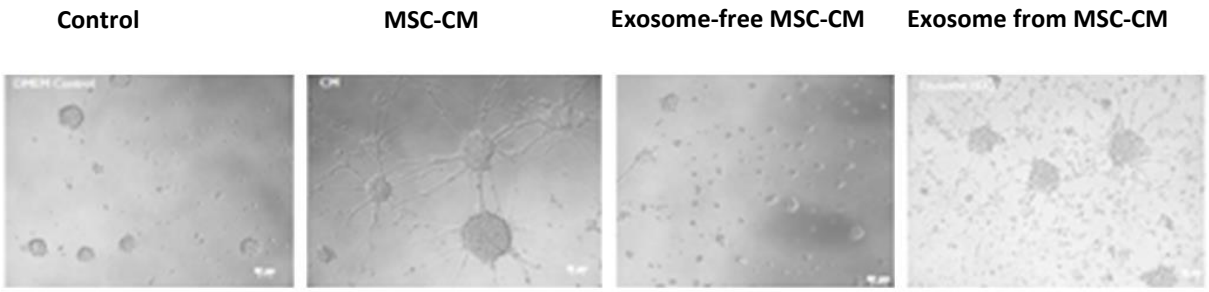


Figure 11: MSC-CM as well as MSC-derived exosomes induce profound morphological changes in GSC. GSC cultures grow as spheroids under control-conditions (Control panel shows representative phase contrast image of GBM cells maintained under stem-like conditions in vitro); upon exposure to MSC-CM from MSCs grown in serum-free conditions (MSC-CM) GSC spheroid cultures undergo large morphological changes within 48h, i.e. spheroid attached and single cells migrating away from the spheroids. However, the morphological changes induced by MSC-CM were not visible when exosomes were depleted from MSC-CM by ultracentrifugation (Exosome-free MSC-CM). When GSCs are maintained in MVs purified from MSC-CM from serum-free conditions, they undergo pronounced morphological changes (Exosome from MSC-CM).

My next aim was to explore if exosomes contain molecular factors that induce the migratory behavior observed in GBMs which eventually contribute to increased GBM invasion. In the biclustering analysis for co-regulated pathways, there were no genes with an apparent potential to mediate cell motility. However, MSCs exert their effects not only via soluble factors but also via exosomes which carry signaling molecules with pronounced pathological

implications (Baglio et al., 2012). Those signaling molecules include mRNAs that can be translated by the recipient cells which eventually lead to cellular changes. However, although the amount of such intravesicular mRNAs is extremely low, much lower than those produced in a cell, they trigger vast variety of pathological outcomes [Hood et al., 2011; Zhang et al., 2011; Roodhart et al., 2011]. So, I needed a new way of investigating such small quantities of genetic materials in exosomes and the transfer from MSCs to GBMs via exosomes. Therefore, I obtained expression profiles, by RNASeq, from GBM cells stimulated with exosomes, GBM cells under control conditions, and the exosomes derived from MSCs (Figure 12).

Such analyses of genome-wide multiple comparisons often generate large sets of differentially expressed genes which might prove rather complicated to handle. However, some relevant genes can be identified in such bundles of data by comparing multiple samples in line of the question in hand. Therefore, I compared differentially expressed genes in exosome-treated GBM13 and control GBM13, using a cutoff value of 1,5-fold change in expression and narrowed down the number of genes to a manageable size. Moreover, I anticipated that the factor responsible for such morphological changes should be an mRNA with an annotated function which is carried within exosomes as most of the sequences emerged from these statistical analyses were epigenetic modulators corresponding to largely unknown pathways (Figure 13).

Consequently, I was able to narrow down my search of pathologically relevant genes greatly by combining these data from microarray data where I identified QKI as a target for increase in viability and RNASeq data from MV-stimulated GBM13 (Figure 4, Figure 12 and Figure 13). As a potential target responsible for those effects, I designated ephrin ligand 3 (EFNA3). Because, EFNA3 levels were very low in the control GBMs, but enriched up to 1,9-fold in exosome-stimulated GBMs and numerous in exosome cargo with 7-fold, compared to controls.

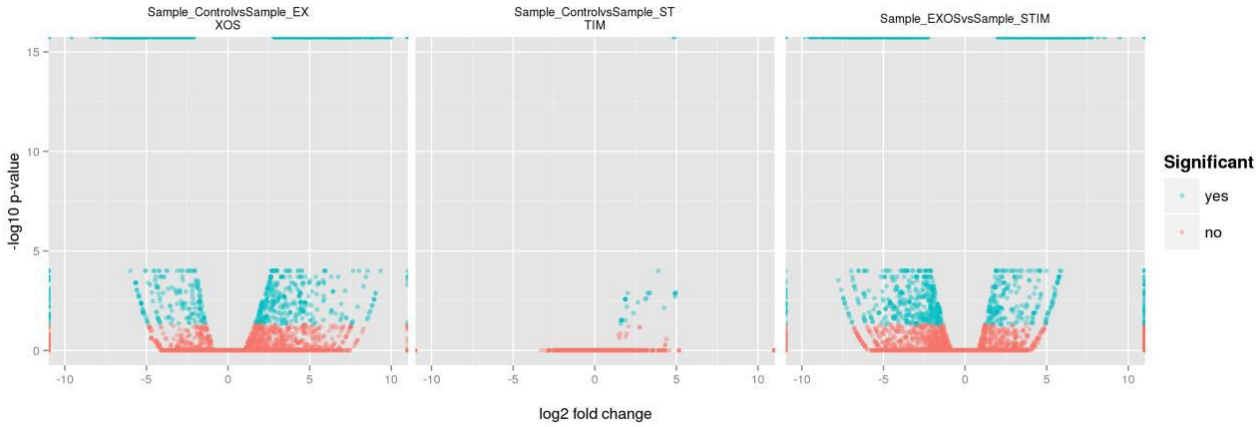


Figure 12: Gene expression profiling by RNASeq identified mRNAs in MSC-MVs which contribute to modulation of gene expression changes in recipient GBM cells. GBM13 cells were maintained under control conditions (Control) or treated with MSC-derived exosomes (STIM). Those cell fractions as well as exosomes alone were then analyzed by RNASeq. All samples were compared with each other Left Panel – Control vs Exosomes; Middle Panel – Control vs MV-Stimulated GBMs; Right Panel – Exosomes vs MV-Stimulated GBMs) and the identity of differentially expressed genes (one dot represents one gene) as well as fold-changes in gene expression (plotted on the x-axis) were obtained. Significant changes were statistically tested (y-axis) and altered gene expression levels for each identified gene (blue dots) were retrieved. Stimulation of GBM cells with MV produced only a relatively small number of altered genes (central figure).

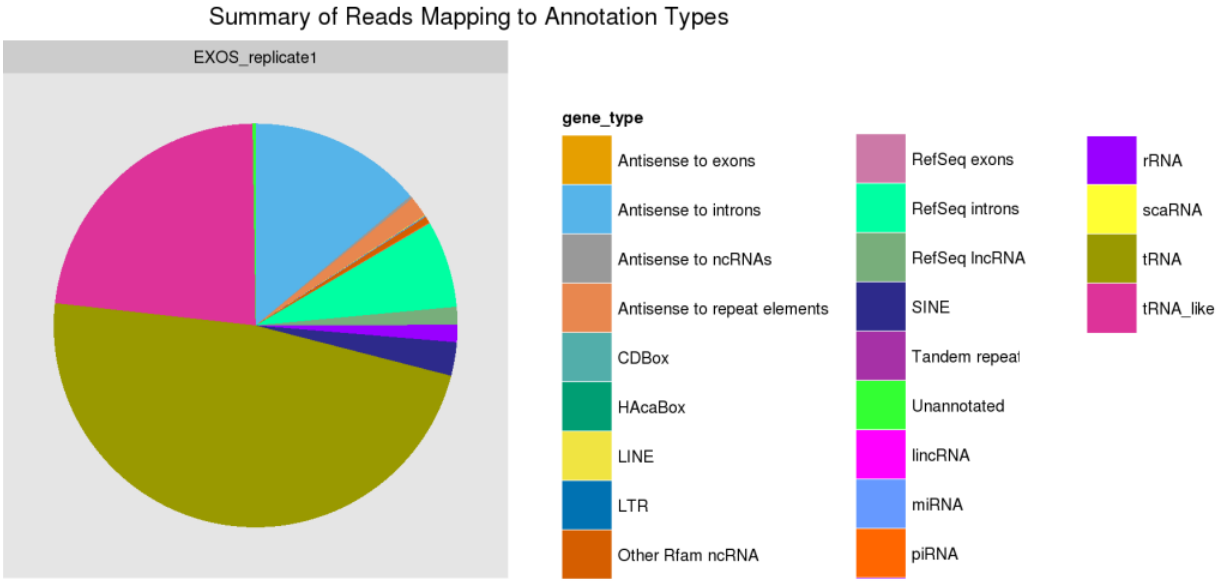


Figure 13: The pie-chart for RNA reads and respective annotations from exosomes alone. The RNAs analyzed in the exosomes were classified based on functions. mRNAs (RefSeq exons) were low in numbers but tRNAs dominate the overall reads. Additionally, epigenetic modulators corresponding to largely unknown pathways were found in exosomes.

4.10. EFNA3 Mediates the Morphological Change / Adherence of GBMs

Previously, RNASeq screen proved EFNA3 to be a relevant target. Hence, I manipulated the EFNA3 in MSCs, which is its source. I was able to reduce the expression levels of EFNA3 in MSCs up to 50% (Figure 14).

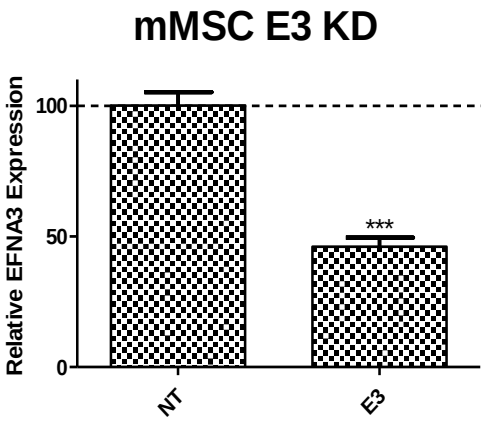


Figure 14: EFNA3 knockdown in MSCs. The knockdown was achieved by a decrease of 52% with E3 construct (among 2 others – not shown). statistical significance is shown *** $p < 0.001$

I subjected GBMs to MSC-CM derived from MSCs that are either EFNA3 knockdown (E3) or transduced with non-targeting ShRNA control vector (NT). Then I quantified the adherent GBM percentage for different GBM lines, keeping in mind that the adherence was the first indication of above-mentioned morphological changes. GBMs cultivated in CM from MSCs with EFNA3 knockdown (E3) displayed significantly reduced adherence compared to those cultivated in CM from control MSCs (NT), shown for GSC lines GBM13, Line 6, Line 9 and Line 11 (Figure 14b). Expectedly, different GSC lines differ in their percentage of adherence, most probably attributable to their different subtypes and genetic backgrounds.

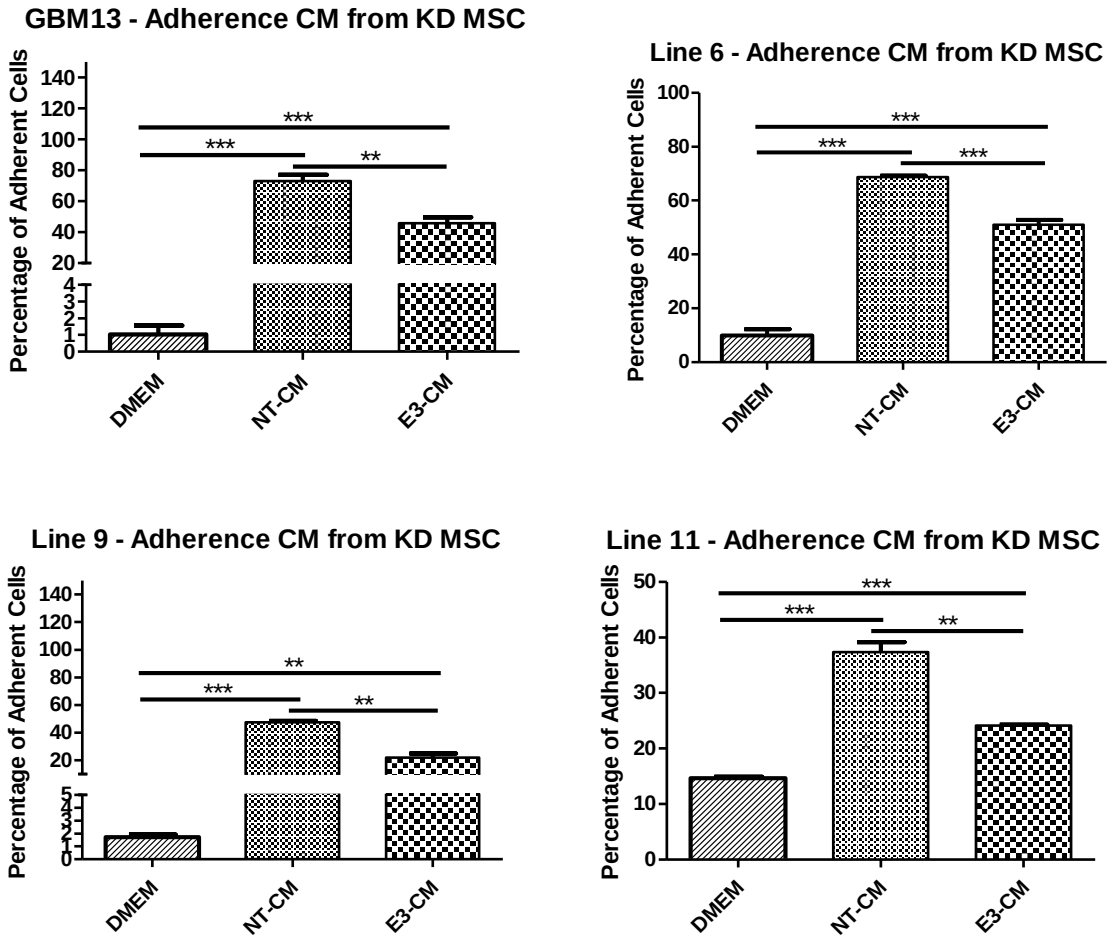


Figure 15: EFNA3 knockdown in MSCs decreases the adherence of GBMs upon CM exposure. The GBMs were exposed to CMs from MSCs with EFNA3 knockdown (E3-CM) or CM from control MSCs (NT-CM). GBMs stimulated with E3-CM displayed a significantly reduced adherence compared to control, NT-CM. This decrease was 25% for GBM13, 20% for Line 6, 20% for Line 9 and 17% for Line 11 compared to vehicle control (NT). It is noteworthy that these GBM lines have different adherence levels at basal conditions (in DMEM). statistical significance is shown *** $p < 0.001$, ** $p < 0.005$, * $p < 0.05$

4.11. EFNA3 promotes the Migration of GBMs

In the previous section I demonstrated that the percentage of adherent GBM cells was decreased upon cultivation in CM from MSCs with EFNA3 knockdown. Additionally, I reasoned that those morphological changes starting with adherence are reminiscent of cell migration and adherence is the pre-requisition of migration in vitro. Therefore, now I investigated if EFNA3 knockdown in MSCs cause a decrease in the migration of GBMs. For this purpose, I developed a migration assay where I plated a single spheroid in each well of a 96-well plate and quantified the occupied area of GBMs over 3 days normalizing it to the initial spheroid

area to be able to precisely detect the changes in migrated area each day (See methods for a detailed methodology). I cultivated spheroids in either CM from MSCs with EFNA3 knockdown (E3-CM) or control MSCs (NT-CM). I found out that, EFNA3 knockdown in MSCs significantly reduced the occupied area which is an indication of the migration, and thereby the invasive behavior of GBMs compared to control, NT and DMEM (Figure 15). The migrated area, however, varies among different GSC lines, so is the reduction in migrated area due to EFNA3 knockdown in MSCs.

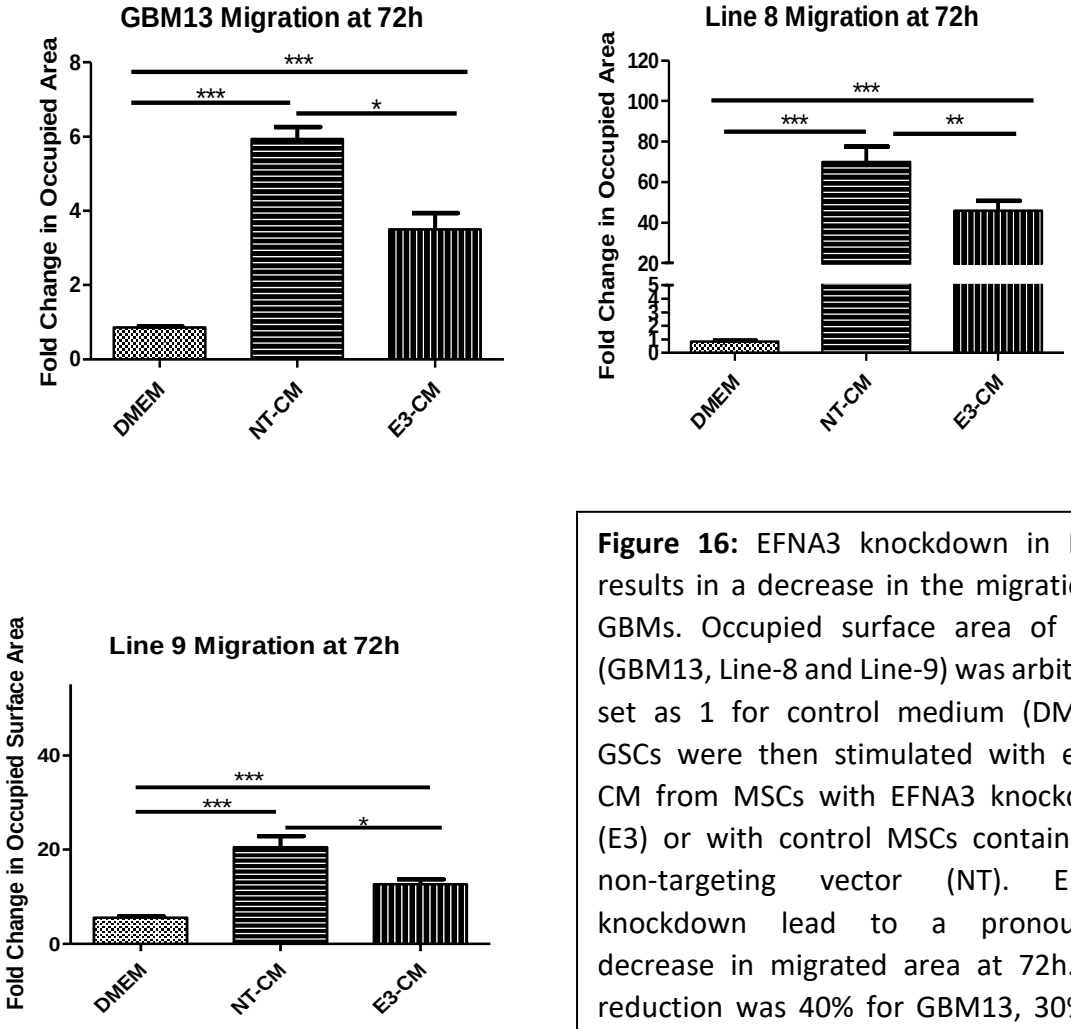


Figure 16: EFNA3 knockdown in MCSs results in a decrease in the migration of GBMs. Occupied surface area of GSCs (GBM13, Line-8 and Line-9) was arbitrarily set as 1 for control medium (DMEM). GSCs were then stimulated with either CM from MSCs with EFNA3 knockdown (E3) or with control MSCs containing a non-targeting vector (NT). EFNA3 knockdown lead to a pronounced decrease in migrated area at 72h. The reduction was 40% for GBM13, 30% for Line-8 and 32% for Line-9, compared to NT. Statistical significance is shown *** $p < 0.001$, ** $p < 0.005$, * $p < 0.05$

In line with this, I next questioned if overexpression of EFNA3 induces an increased migration in GSCs. Therefore, I overexpressed EFNA3 in MSCs up to 7.2 fold (Figure 17)

Next, I cultivated the same GSCs as above (GBM13, Line-8 and Line-9) in CM from EFNA3-overexpressing MSCs (mEFNA3-OE) and control MSC (Empty). I observed that, EFNA3 overexpression lead to a moderate increase in the migrated area for GBM13 alone, but not for Line-8 and Line-9 (Figure 18) which could be attributed to a saturation of migratory behavior at the basal level of EFNA3 expression, in the latter.

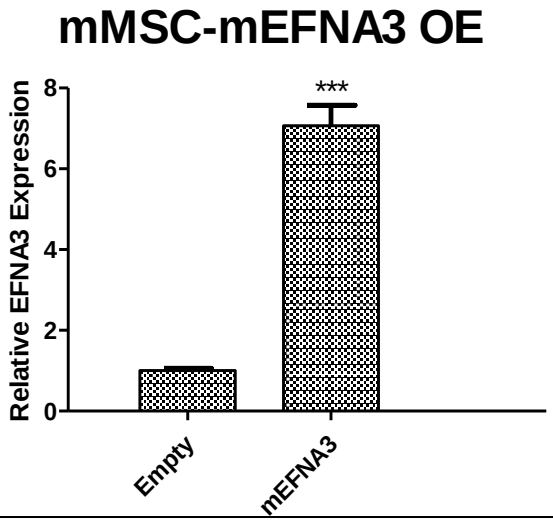


Figure 17: EFNA3 overexpression in MSCs. In order to overexpress EFNA in MSCs, I transfected them with an overexpression vector encoding EFNA3 sequence (see methods for more detailed information about the vector used) and overexpression was assessed by quantitative real time PCR using TaqMan probes which demonstrated an increase of 7.2-fold in EFNA3 expression. Data were indicated as Mean ± SD. Statistical significance is shown *** $p < 0.001$

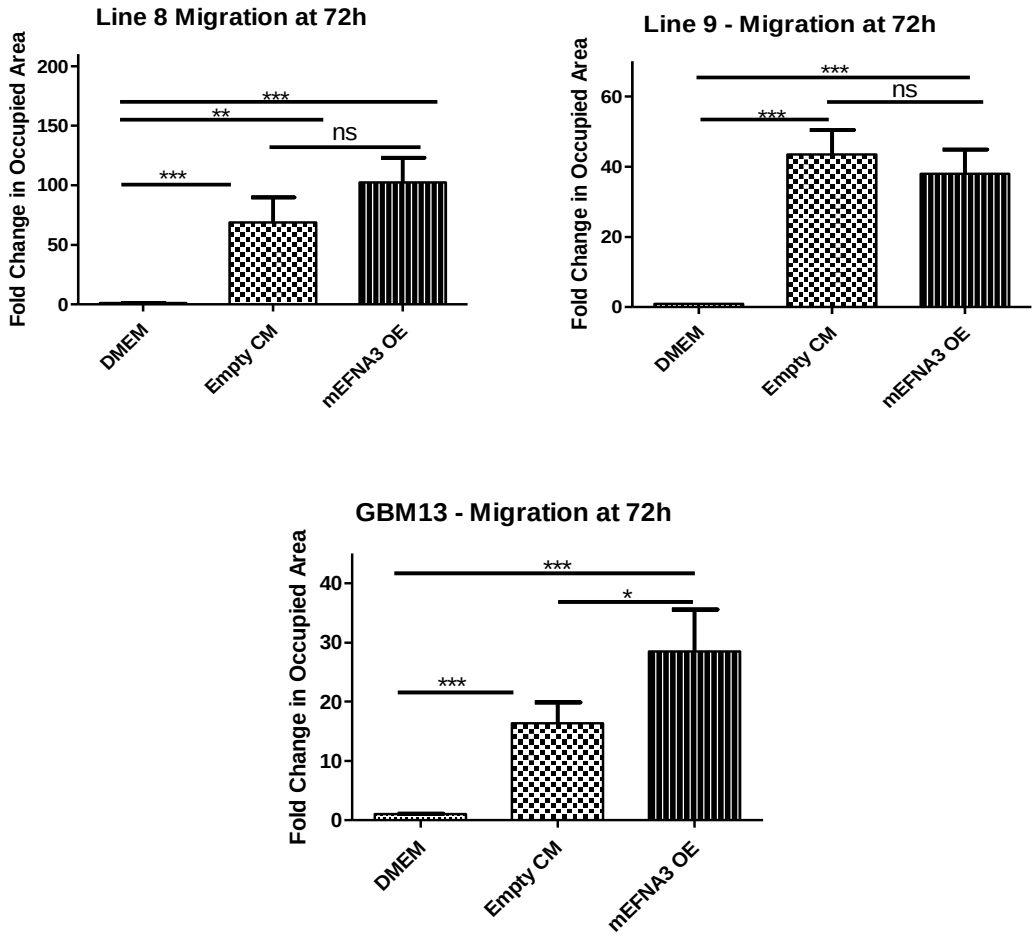


Figure 18: EFNA3 overexpression in MCSs results in a moderate increase in GBM13 but not in other GSCs. Occupied surface area of GSCs (GBM13, Line-8 and Line-9) was arbitrarily set as 1 for control medium (DMEM). GSCs were then stimulated with either CM from MSCs with EFNA3 overexpression (mEFNA3 OE) or with control MSCs containing an empty vector (Empty CM). EFNA3 overexpression lead to a moderate increase in migrated area for GBM13 alone, at 72h, compared to control NT. The increase was 42% for GBM13. Statistical significance is shown *** $p < 0.001$, ** $p < 0.005$, * $p < 0.05$

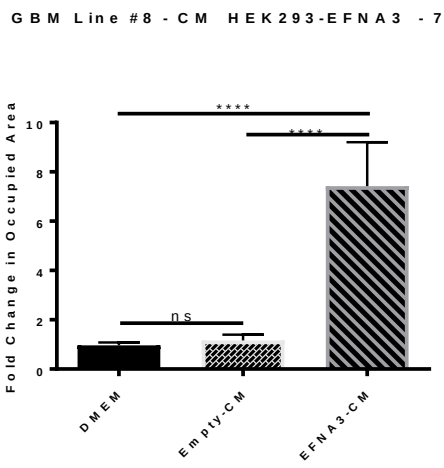


Figure 19: GSC migration is induced when EFNA3 is overexpressed in HEK293 Cells. HEK293 cells have no detectable expression of EFNA3. CM from HEK293 cells overexpressing EFNA3 or containing an empty vector was produced in serum-free conditions and GSC migration was measured over the course of 72h. CM from HEK293-EFNA3-overexpression dramatically induced migration of GSCs (Line-8) while CM from HEK293-Empty did not induce any migration. Data were presented as Mean ± SD. Statistical significance was shown; **** p<0,0001

As an alternative approach for proving the role of EFNA3 in migration, I overexpressed EFNA3 in HEK293 cells, which have no apparent expression of EFNA3 and release vast amounts of exosomes. I produced serum-free CM from EFNA3-overexpressing HEK293 (EFNA3-OE) cells or those carrying an empty vector (Empty) and applied on GSCs. CM from EFNA3 overexpressing HEK293 cells induced a significantly increased migration of GSCs, compared to control DMEM and Empty-CM (Figure 19).

In the previous sections, I demonstrated that EFNA3 mRNA is carried from MSCs to GBMs within exosomes. In turn, EFNA3 mRNA induces the migration via an unclear mechanism. Knocking down the EFNA3 in MSCs resulted in a marked decrease of the migration of GBMs upon exposure to CM (serum-free). In order to strengthen this finding with additional proves, I expressed the knockdown ShRNA in GSCs to eliminate the endogenous EFNA3 as well as the EFNA3 mRNA delivered by the exosomes from MSCs. Moreover, I stimulated EFNA3 knockdown GSCs with either CM or exosomes purified from MSCs. Elimination of exosome-delivered EFNA3 in GSCs (#9-E3) lead to a pronounced decrease of the occupied area compared to control (#9-NT) when stimulated with exosomes. Moreover, the EFNA3 knockdown completely abrogated the effect of CM stimulation in terms of migration (Figure 20).

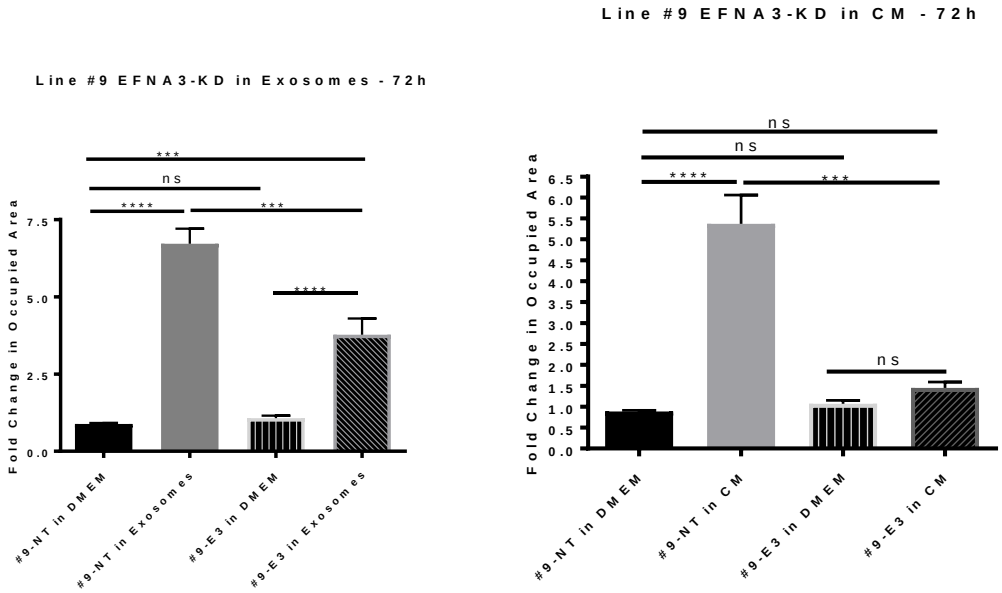


Figure 20: Knockdown of EFNA3 in GSCs have profound effects on migration. EFNA3 knockdown construct (E3) was expressed in GSCs to knockdown of endogenous EFNA3 mRNA and decrease the effects of exosome-delivered EFNA3. GSCs were stimulated both with CM (left graph) and exosomes purified from MSCs (right graph). Knockdown of both endogenous and exosome-delivered EFNA3 (#9-E3) caused a marked decrease of the occupied area, hence migration, of GSCs when stimulated with exosomes compared to control (#9-NT), but completely blunted the effect of CM stimulation (left graph - #9-E3 in CM). Data were presented as Mean ± SD. Statistical significance is shown *** p<0,001; **** p<0,0001.

4.12. Message Transfer via Exosomes from MSCs to GBMs

In the previous sections, I demonstrated that, EFNA3 mRNA is transferred from MSCs to GBMs via exosomes. Now I wanted to prove the concept that, MSCs package functional mRNAs into exosomes and this "message" is translated and activated in GBMs. Moreover, we do not know how frequently this transfer of mRNA via exosomes takes place in vivo. Additionally, it is pivotal to track individual cells receiving exosomes from MSCs in terms of metastatic infiltration to uncover specific role of the transfer of mRNAs. Therefore, I generated a reporter system by expressing Cre-recombinase in MSCs and a floxed-flipped-GFP in GBM13 in order to permanently label the GBMs which had taken up exosomes from MSCs (Figure 11 – upper panel). It was previously shown that Cre-recombinase is packaged in exosomes, taken up by target cells (In our case GBMs) and expressed into functional protein product and activates the expression of flipped-GFP (Ridder et al, 2012). To test the functionality of the system, I co-cultured this transgenic MSCs and GBM13 cells. After 72h, I was able to detect GFP-positive GBMs in co-culture conditions but not in control (GBM13 alone), which is an indication of Cre-mRNA transfer from MSCs to GBMs (Figure21– lower panel). However, this observation was not very frequent, so we do not know if this system could be used in vivo at this stage. Improvements are needed before in vivo studies.

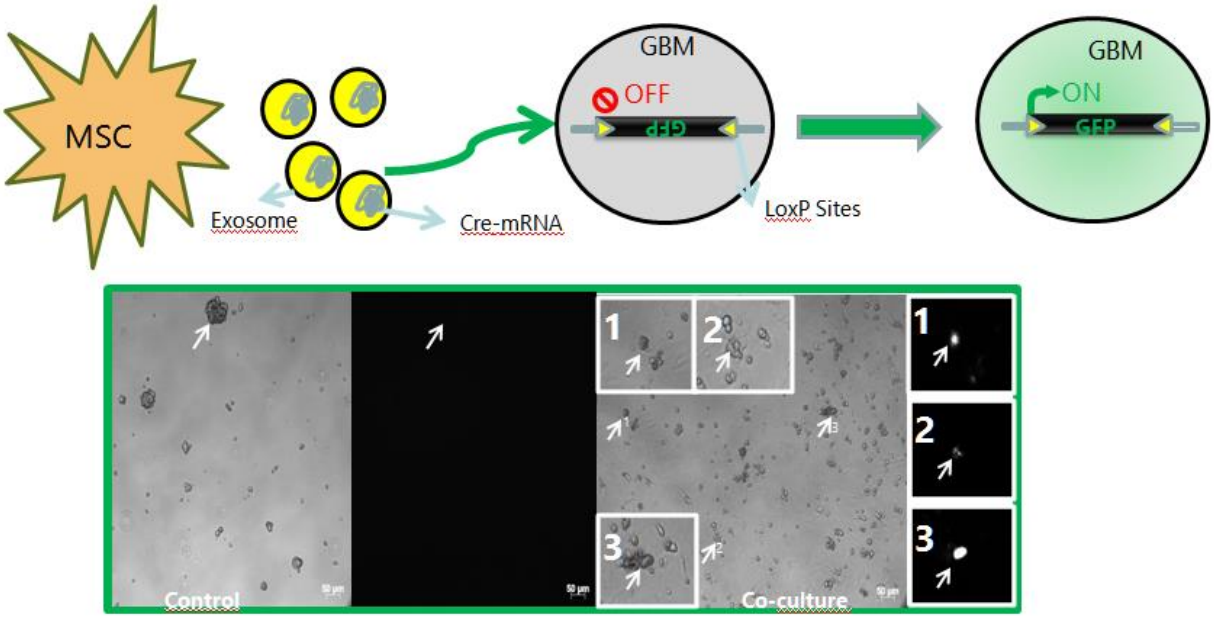


Figure 21: mRNA exchange between MSCs and GBMs in vitro. MSCs were stably transfected with Cre-recombinase expression vector, and GBMs were stably transduced for expression of an inactivated flipped form of GFP. Then those two were cultivated together. This resulted in "turning on" of the GFP expression in GBMs. This proves the transfer of Cre-mRNA from MSCs to GBMs. Three white rectangles show independent areas where GBMs have taken up exosomes carrying Cre-mRNA (depicted as 1,2,3). GBM cells with activated GFP expression upon taking up exosomes from MSCs expressing Cre-recombinase are indicated with arrows. Scale bars are 50 um.

4.13. Tumor Satellite Model

From the initial in vivo experiments, I concluded that the MSC-GBM interaction takes place at the tumor satellites, instead of the main tumor mass. Later, the in vitro experiments mimicking the conditions that are present at the tumor satellites demonstrated that the MSCs have pro-tumorigenic effects on GBMs such as increased viability and chemoresistance (mediated by QKI) and invasion (mediated by EFNA3 transferred by exosomes) which overall augment GBM relapse. Therefore, it is pivotal to study those interactions in vivo. In order to test this hypothesis, we would need a relevant in vivo model where we could recapitulate the standard series of treatment. However, it is very difficult to perform tumor resection in a mouse brain due to its small size and alternative glioma models for studying tumor relapse are not wide-spread. That's why I tried to establish such a model which allows for reproducible mimicking of tumor debulking. In GBMs, I overexpressed thymidine kinase (TK), which functions as a suicide gene upon application of the prodrug Ganciclovir (GCV) by converting it into a cytotoxic residue inducing cell death. For this, I used TK-expression vector, normally used for gene-

therapy purposes (by Hrvoje Miletic, University of Bergen, Norway). These GBM cells expressing TK were inoculated in mice and after tumor growth of 7 days, intracerebral osmotic minipumps loaded with 2 mM GCV were implanted. This experimental model allowed for 95% reduction of tumor size on day 21 (Figure 22).

As an alternative approach, I sought to infuse GCV intravenously, instead of using osmotic minipumps. The advantage is that, this method is broadly applied without any advanced surgical skills, does not add another experimental parameter (the effects of osmotic pumps are required to be controlled) and can be combined with other therapeutic treatments easily. Therefore, I recapitulated the above-explained experiment with several experimental groups of mice including relevant controls for proper glioma growth without GCV treatment, for GBMs without TK expression in the presence of GCV along with experimental situation where GBM cells expressed TK and GCV was injected to reduce the size of the tumor mass. Intravenous injection of 25 mg/Kg body weight/animal/day GCV resulted in a similarly reduced tumor mass (Figure 23)

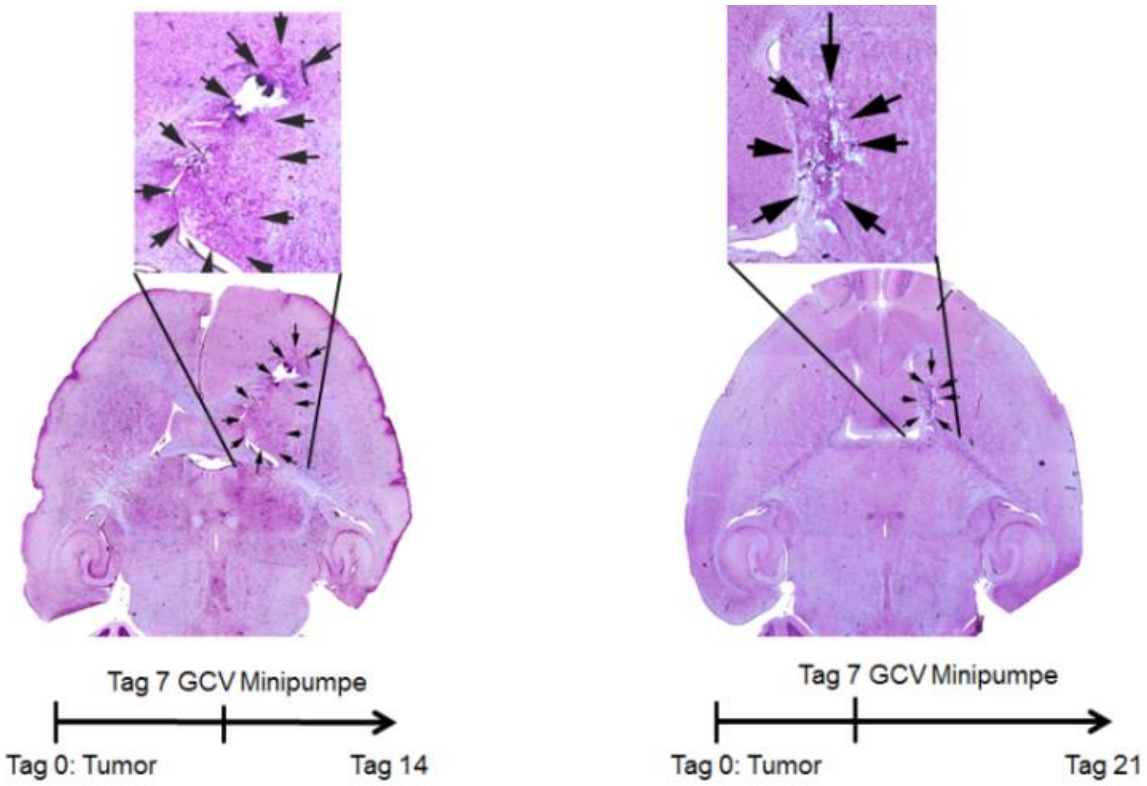


Figure 22: GBM13 cells (of human origin) expressing TK were implanted into immunocompromised mice. After 7 days of tumor growth, 2 mM GCV was infused via osmotic minipumps for 7 days (left panel) and for 14 days (right panel). GCV treatment massively reduced the size of the main tumor mass, which was not observed in the negative controls such as TK-free GBMs with GCV infusion and TK-expressing GBM receiving saline infusion (not shown).

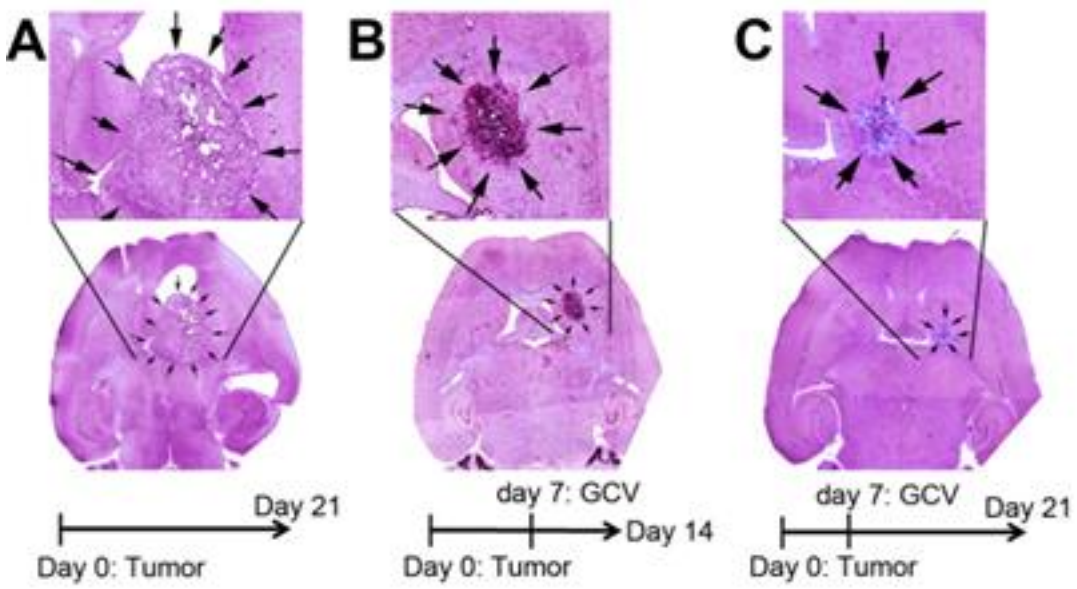


Figure 23: GBM cells (the human GSC GBM13) expressing TK were implanted into immune-compromised mice. Tumor growth was allowed and different concentrations of GCV were applied to different experimental groups of mice. Data are presented from one experimental cohort receiving 25 mg GCV / kg body-weight / animal / day. Experiments were performed with n = 4 mice per time point (16 mice per experimental group + 4 additional controls) and in a total of 3 different cohorts receiving different amounts of GCV (60 mice in this experiment). **(A)** GBM-13 cells expression TK formed histopathologically detectable gliomas within 21 days in absence of GCV. **(B)** Short-term GCV treatment (starting 7 days after glioma inoculation and ending 14 days after glioma injection) indicated a reduction in tumor mass. **(C)** Gliomas were massively shrunk after 14 days of GCV application.

4.14. SUMMARY

GBMs have proven to be a major pathology with vast infiltration potential and extreme chemo- and radio-resistance leading to devastating outcomes. The GBMs, however, are not only detrimental on the host alone but also they interact with the microenvironment to manifest some of their pathological hallmarks. Within the tumor microenvironment, MSCs have gained a strong attention in the recent years. The role of MSCs within the brain tumor niche has been partially explained including pro- and anti-tumorigenic effects. It was, however, a controversial issue as to what makes MSCs pro- or anti-tumorigenic. Moreover, the contribution of MSCs in the brain tumor histopathology was not yet fully uncovered.

Here I investigated the role of MSCs in the brain tumor pathology and the signaling mechanisms. It was shown earlier in our lab that MSCs act as anti-tumorigenic in the presence of serum and anti-tumorigenic in serum-free conditions. Which condition is more relevant to the pathological situation was an open question. When I co-inoculated MSCs with GBMs, they homed the tumor satellites, where they are probably not in contact with blood-borne factors. Therefore, I concluded that serum-free conditions are more relevant to in vivo pathological situation.

MSCs promoted viability of many primary GSC lines under serum-free conditions. The induction of survival and proliferation was mediated by the increase in the levels of QKIs in GBMs upon exposure to MSC-derived soluble factors. In turn, QKIs increase the levels of EGFR causing an overresponse to growth factors. Moreover, increased QKI-levels in GBMs mediate the chemoresistance against TMZ. In addition to soluble factors, MSCs signal via exosomes. Those exosomes carry EFNA3 mRNA from MSCs to GBMs. EFNA3 expression in turn induces cellular migration. The inhibition of EFNA3 transfer via genetically engineered antibodies against exosome docking sites on the recipient GBM cells or against EFNA3 (if it is translated and integrated on the surface) as well as EGFR-blockade might prove useful for future therapeutic approaches against GBMs.

There are no well-established mouse models to study the interaction between MSCs and GBMs at the tumor satellite. Therefore, I sought to establish such a model by expressing HSV-TK in GBMs and inducing cell death via GCV administration either by osmotic pumps or via systemic injection and established a model where one can study tumor satellites. Additionally, I established a model to study the mRNA transfer from MSCs to GBMs. For this, I expressed Cre-recombinase in MSCs, and flipped- and floxed-GFP in GBMs. The Cre-recombinase mRNA is packed into exosomes and delivered to GBMs, labelling GBMs with GFP for the rest of their lives. This enables us to demonstrate the transfer of mRNA as well as to track individual GBM cells, contacted by the MSCs, in terms of their migratory behaviors in vivo. All in all, I uncovered a previously unknown action of MSCs in GBM pathology defining two targetable systems (EGFR and EFNA3) and established two models to study in vivo interaction of MSCs with GBMs.

5. DISCUSSION

5.1. MSCs Continuously Home Tumors

MSCs have the ability to locate and integrate within the tumor tissue, which is termed as "homing". This homing behavior is guided via some tumor-specific receptors and soluble tumor-derived factors like SDF-1, TNF-α, and interleukins. Once they reach the tumor, they perform either pro- or anti-tumorigenic roles which include, but not limited to, immune response suppression [Djouad et al, 2003], inhibition of apoptosis in tumors [Ramasamy et al, 2007], stimulation of epithelial to mesenchymal transition (EMT) [Martin et al, 2010], tumor cell proliferation [Liu et al, 2011], angiogenesis [Hung et al, 2005], tumor cell migration [Corcoran et al, 2008], invasion [Corcoran et al, 2008] and metastasis [Karnoub et al, 2007].

GBMs exhibit a hard vascularization containing incomplete circulation with open-end blood vessels, causing leakage similar to all tumors. In this respect, tumors are termed as wounds that do not heal. MSCs are thought to reach brain tumors via those blood vessels. As the tumors are called wounds that do not heal, MSCs act as if they are healing wounds. MSCs have two phases of injury response called early and late response. In early injury response MSCs induce pro-inflammatory pathways in the injury site to promote clearance of the wound from infectious agents. In the late injury response, MSCs induce local stem cells for scar formation, tissue repair and eventually wound healing. Early response could be relevant to the anti-tumorigenic roles of MSCs. Tumors have the ability to suppress immune cells and corrupt them for their benefits. By elevating immune response, MSCs increase the tumor cell clearance by the immune system. Late response might explain the pro-tumorigenic effects of MSCs. They secrete factors that stimulate local stem cells to proliferate for wound healing, which triggers cancer stem cells to proliferate as well.

Furthermore, by different research groups, MSCs have been shown to differentiate into different stromal cell types and support the tumor in a variety of ways, such as differentiating into Cancer-associated fibroblasts (CAFS) in peripheral tumors [Gao et al, 2009]. Djouad et al, in 2003, also demonstrated that MSCs do not require an integration into the tumor stroma in order to perform the afore mentioned roles but they are able to stimulate similar outcomes in terms of tumor pathology with paracrine signaling [Djouad et al, 2003]. Roodhart et al in 2011 also showed that MSCs can also mediate and support chemoresistance in tumors where they made a modelling experiment mimicking the mobilization and integration of MSCs into tumor stroma [Roodhart et al, 2011].

In this study, I tested the potential of MSCs locating GBM xenografts in vivo. I inoculated MSCs following GBM inoculation and tumor formation. The MSCs were inoculated at a distance of 1.5 µm from GBM tumor mass. Only very few MSCs were observed in the main tumor mass, whereas ratio of MSCs to GBM cells in the satellite tumor was close to 1:1. This finding is consistent with the observations made by others where MSCs were more successful at homing satellites than the main tumor mass [Bexell et al., 2009]. This phenomenon could be therapeutically important because main tumor mass is surgically removed with ease while satellites are mostly out of reach and cannot be easily detected with conventional noninvasive

methods until they grow up to a certain size [Tilghman et al., 2014]. Using MSCs as tools, or targeting their interactions with GBM satellites may prove a useful means of therapy development. However, mouse brain is too small for noninvasive surgical operations and not many alternative tumor models exist for studying satellite tumors. To address this problem and explore the MSC-GBM satellite interaction in vivo further and to develop potential therapeutic approaches, I developed a tumor satellite model where I used a suicide gene in GBM cells which can be triggered with GCV infusion, eliminating a majority of GBM cells leaving behind small clusters of cells that develop into satellite tumors later. In the early experiments where I tested this system, I could achieve satellite tumor formation upon GCV infusion via osmotic pumps, or intraperitoneal injection. The size of the tumor was dependent on the duration of the GCV application, i.e. more prolonged application of GCV lead to smaller tumor sizes, 14 days being the ideal time for a satellite-size tumor to form. This system can be improved with later studies to test the precision and relevance to the pathogenesis of the GBMs in patients.

5.2. MSCs Have Profound Effects on GBMs

We demonstrated that, MSCs cultivated in the presence of FCS have different physiological effects on GBMs than those maintained in FCS-free medium. This difference was evident in the GBMs treated with MSC-CM from both MSC-cultivation conditions. This finding supports the finding that MSC secretome varies based on the conditions they reside in [Vizoso et al., 2017]. When MSCs integrate into the tumor stroma in satellites, they are no longer in contact with the blood stream unlike they used to be in the bone marrow (and in main tumor mass which exhibit a large angiogenesis [Brat et al., 2003]) as satellite tumors do not possess a hard vascularization. This means, they are no more able to receive stimulation from blood borne stimulants such as chemokines and growth factors. In order to mimic the exact same situation, we cultivated our MSCs in the absence of FCS which contains the similar or the same stimulants of blood stream.

5.3. MSC-CM Effect is Different for Different Cell Lines

When MSCs were cultivated in serum-free medium and this CM was applied onto GBMs, the viability of GBMs was significantly increased. The extent of this increase in viability of GBMs varied greatly among different lines. Moreover, some cell lines didn't even respond to CM in terms of increase in the viability. This could be due to cell-intrinsic properties which varies from cell to cell. The GBMs have been attributed to three different subtypes which are defined by the gene expression patterns [Verhaak et al., 2009]. In addition to these different gene expression patterns, the GBM lines I used were primary GBMs derived from patients, therefore they possessed different genetic backgrounds, too. In the normal growth medium, they displayed different growth patterns as well as different morphological properties. Given these genetic and physiological variations, the CM from MSCs might have different potencies for each cell line. Proneural GBMs have been shown respond CM with the highest increase in the viability, whereas mesenchymal GBMs displaying the lowest response in terms of increase in viability. Additionally, even each cell line that belongs to the same subtype has differences in terms of the degree of increase in viability, demonstrating the complexity of the interaction between MSCs and GBMs.

5.4. Viability Increase by MSC-CM is Mediated by the Soluble Factors in CM and the QKIs in GBMs

I then investigated the cause of the increase in the viability by MSC-CM both from the MSC side and the GBM side. So, I sought answers to two questions: What do MSCs release into the medium that leads to an increase in the viability of GBMs? How do GBMs respond to the cues they receive from MSCs via CM? MSCs interact with other cells either via soluble factors they secrete or exosomes packed with signaling molecules which are released to the vicinity. I showed that the viability increase by the CM is mediated via the soluble factors that are secreted from MSCs. This was evident when the exosomes alone were not able to boost the viability of the GBMs but the CM devoid of exosomes showed an increase in the viability at a comparable level to that of native CM.

To investigate the intracellular gene expression patterns in GBMs upon CM stimulation, a co-regulated pathway analysis via a biclustering method was performed [Cheng and Church 2000]. Upon statistical analyses and filtering relevant genes, QKIs emerged as candidates for the effect of MSC-CM on GBMs. Because, when global gene expression patterns were mapped comparing controls to CM-treated GBMs, activities of certain pathways shift further from the control in a time-dependent manner and QKI is at the central nodule of those pathways. Moreover, QKIs have been implicated in the tumor development as both pro- and anti-tumorigenic. QKIs also have roles in cell cycle regulation [Biedermann et al., 2010]. Additionally, QKI-5 is implicated as a tumor suppressor and Fu et al. showed that QKI-5 has anti-tumorigenic effects via suppression of cyclin-D1 and in turn the MAPK pathway [Zhao et al., 2010; Fu et al., 2015]. Furthermore, Chen et al. discovered that, Quaking suppresses tumor growth via targeting specific miRNA via p53 pathway [Chen et al., 2012]. There are few studies showing pro-tumorigenic effects of QKIs [Wang et al., 2013; Bandopadhayay et al., 2016] and one publication is demonstrating QKI suppression by MiRNAs leading to tumor progression [Pillman et al., 2018]. In light of these previous research findings, I predicted that QKIs have pro-tumorigenic roles in brain tumors [Wang et al., 2013; Bandopadhayay et al., 2016], or some undiscovered pathway could be the mechanism by which the viability and sphere formation increases.

In order to test if QKI is important for the increase in viability and proliferation, I used gene manipulation strategies. I showed that overexpression of QKIs, for isoforms QKI-5, QKI-6 and QKI-7, results in a marked increase in the viability of GBMs to the levels comparable to that mediated by CM. This increase in viability was, however, most prominent with elevation of QKI-6 expression. Additionally, in one line (Line 7) the knockdown of QKIs blunted the effect of MSC-CM in terms of viability increase. On the other hand, I carried out those overexpression experiments for QKIs only in classical GBM Line 4 and proneural GBM Line 7, as the increase in the viability was largest in those two lines, and they were more readily available than the other GBM lines, which is an important criterion for performing parallel experiments. Therefore, the overexpression of QKIs in mesenchymal subtype (the increase in viability of mesenchymal subtype was less compared to other subtypes) could lead to a different outcome or the other QKI isoforms than QKI-6 might prevail in terms of the increase in viability.

Furthermore, I showed that the increase in the viability was mediated by the soluble factors in CM, rather than the exosome-cargo. However, I did not explore which soluble factors take part in this phenomenon. Because I had two major directions to take: QKIs or exosome cargo which later proved to be related to invasion and more relevant to therapeutic intervention as they are not intracellular molecules unlike QKIs. Moreover, a body of research has been performed on QKIs. In one particular study, Wang et al. showed that QKI-5 and QKI-6 directly interacts with miRNA7 and sequesters it in the nucleus preventing it from being loaded onto and processed by the RISC complex, in glial cells. This interaction prevents the maturation of miRNA7, which targets EGFR [Wang et al., 2013]. Likewise, the knockdown of QKIs in Line 4 lead to a decrease in the EGFR levels, which is in line with the findings by Wang et al. Therefore, Line 4 uses the same conserved mechanism from the glial cells, in terms of the QKI-dependency.

As mentioned above, a soluble factor mediates the increase in QKI levels and in turn viability and sphere formation. However, what leads to the overexpression of QKIs in GBM cells is still unknown. There might be some soluble peptides that are cleaved from the surface of MSCs and released into the cultivation medium, which in turn acts on a receptor on the GBM surface leading to a series of signal transductions converging on QKI pathways and causing an increase in the QKI levels. It could also be a peptide that is simply released to the medium which in turn acts as a ligand for a receptor. This phenomenon was demonstrated by some research groups where Ephrin ligand is cleaved from the cell surface and acts on Eph receptors at a distant site causing alterations in Eph receptor clustering or abundance on cell surface [Bartley et al. 1994; Hattori et al., 2000; Wykosky et al. 2008]. Similarly, exosome bound ligands are cleaved to become soluble peptides and act on distant receptors [McKelvey et al., 2015].

Alternatively, it could be a fatty acid or a fat compound that is released or deposited as a waste to the medium by MSCs causing a trigger in GBMs for cell division. I subjected CM to high temperature and applied onto GBMs and observed a decrease in the proliferation and viability of GBM cells compared to untreated control CM (Data not shown). Although fat compounds are more heat-resistant than peptides, such high temperature exposure (75 ˚C) is likely to cause distortions in structure causing a less functional or nonfunctional molecule, explaining the phenomenon. In fact, a system governing fatty acids released from MSCs exerting effects in tumors has been demonstrated by Roodhart et al. where they showed that platinum-induced fatty acid release from MSCs confers cisplatin chemoresistance in murine tumors (Roodhart et al., 2011).

5.5. Exosomes do not Cause Viability Increase but the Adherence and Morphological Changes

I showed that, exosomes isolated by ultracentrifuge in a gradient did not yield any increase in viability (even when concentrated), however, CM that lacks exosomes (depleted by ultracentrifugation), boosted the viability of GBMs at a comparable level to native CM containing exosomes. I tried to recapitulate this by isolating exosomes with a commercially available exosome isolation reagent (Exoquick-TC kit). Contrary to the previous finding, however, exosomes caused a significant increase in the viability of GBMs only when they were applied onto GBMs in a concentrated way (5X-10X). This increase in the viability was not at a

comparable level to that of the native CM or EDCM and was completely lost when exosomes were less concentrated (3X or lower). One possible explanation for this observation could be that the Exoquick-TC kit is likely a lipophilic reagent clustering hydrophopic molecules such as cell membrane debris or hydrophobic proteins (along with exosomes) which are the agents that cause the viability increase in the CM. However, the concentration of those factors in exosome pellet is much lower than in CM. That is why, exosome pellet causes a slight increase in the viability when they are concentrated but fail to boost viability when below 3X concentration. Moreover, ultracentrifuge provides more reliable and pure exosomes. Therefore, I concluded the exosomes alone do not have the ability to increase the viability of GBMs.

Additionally, the MSC-CM caused a morphological change in GBMs. The GBMs, which normally grow as floating spheroids, adhered to the plate and had protrusions in two opposing sides of the cells, reminiscent of migrating cells [Hakkinen et al., 2010]. GBM cells treated with the exosomes and CM had morphological changes but the EDCM completely lacked the adherence and the morphological changes. So, the morphology change and adherence were exclusively brought about by the exosomes. Moreover, the morphological changes due to CM and exosomes were observed the most in proneural subtype of GBMs, and the least in the classical subtype suggesting a subtype dependency of the observed phenotype.

5.6. Exosome Cargo Contains and deposits EFNA3 into GBMs

Exosomes are packed with proteins, mRNAs and miRNAs and released to the cell exterior [Mathivanan et al., 2009]. I hypothesized that some miRNAs or mRNAs mediate the morphology change and the adherence of GBM cells based on some publications which demonstrate the message transfer via exosomes. For instance; Tomasoni et al showed in 2013 that MSCs can transfer growth factor receptor mRNAs via exosomes to proximal tubular epithelial cells causing an increase in their proliferation rate [Tomasoni et al., 2013]. Additionally, Ono et al in 2014 demonstrated that exosomes from MSCs carry a specific miRNA which renders metastatic breast cancer cells dormant [Ono et al., 2014]. Therefore, we performed an NGS readout with exosomes, and exosome-stimulated GBM cells. I analyzed the readouts by taking the cutoff value as 1,5X-fold change. From this analysis, a few miRNAs and some mRNAs emerged. EFNA3 has caught our attention, because there is already a body of research which describes the role of Eph-Ephrin signaling and their potential implications in cancer [Fertuga et al., 2014; Merloz-Suarez et al., 2008; Vail et al., 2014]. Seeing in numbers the direct transfer of EFNA3 mRNA from exosomes to GBMs where EFNA3 was abundant in exosomes and the abundance increased in the exosome treated GBMs but not in the vehicle treated ones was a supporting observation. That's why I focused on EFNA3 in MSCs.

5.7. EFNA3 Manipulation in MSCs

EFNA3 knockdown in MSCs was performed with lentiviral vectors. After the selection of transduced MSCs, the amount of knockdown was tested with quantitative real-time PCR. Among three different knockdown vectors, one was selected as the most potent. However, even with the most potent knockdown, the highest amount of knockdown we could reach was 52% reduction in gene expression. The knockdown of EFNA3 in MSCs reduced their growth rate, changed their morphology and decreased the adhesion of MSCs to the plate, which was

not observed in the MSCs transduced with non-targeting control vector. This observation was consistent with the finding that EFNA3 abundance is an important determinant of adherence and survival in MSCs [Alfaro and Zapata, 2018]. Furthermore, some cells in vivo and in vitro, require some contact inhibition in order not to malfunction or to prevent overgrowth. In the developmental processes, EFNA3 is known to be involved in the formation of tissue borders and axonal tracts by repulsive mechanisms [Palmer and Klein 2003]. It might be possible that EFNA3 also regulates the contact inhibition of MSCs and when we knock it down, the MSCs loses the contact inhibition too leading to malfunctioning [Hoffmann et al., 2009]. Overall, a large decrease of EFNA3 in MSCs might be harmful for their survival. This may explain the low amount of decrease in EFNA3 expression in MSCs upon knockdown as the surviving MSCs are those with relatively lower decrease in EFNA3 expression but not enough to cause detrimental outcomes.

The exosomes alone do not lead to viability increase in GBMs when they are not concentrated, so I recapitulated this in terms of the effect of EFNA3 knockdown on viability of GBMs. The CM from MSCs with EFNA3 knockdown caused neither an increase nor a decrease in the viability of the GBMs compared to the non-targeting control (data not shown). This observation supports my hypothesis that EFNA3 in MSCs is not related to the increase in the viability, but it is related to the adherence and the morphological changes which potentially are related to a migratory behavior.

5.8. EFNA3 Mediates the Migration of GBMs

The effect of EFNA3 transfer from MSCs to GBMs within exosomes was observed as morphological changes (adherence and protrusions). Moreover, the percentage of the adherent GBM cells was reduced upon application of CM from MSCs with EFNA3 knockdown. Therefore, the effects of EFNA3 knockdown in MSCs on the GBM migration was tested. In order to do this, we first decided to plate a single or a few spheres in a well of a 96-well plate and measure the distance between the center of a sphere and the cells migrated furthest from the sphere center. However, plating more than one sphere posed a high ambiguity as migrating cells from different spheres mixed in with each other. Additionally, measuring the distance from the sphere center to the furthest-migrating cells also caused a problem with the normalization. The migrated distance was different for each sphere, proportional to the sphere size. Moreover, not all the spheres had the same migration pattern, such that some cells migrated as a thin line and migrated far, whereas some cells migrated not too far but spread in a larger area. So, the linear distance measurement didn't fit to our purpose as it didn't give us a reliable measurement of migration that can reflect the real difference between GBMs treated with CM from control and EFNA3-knockdown MSCs. That's why I came up with the proportional measurement of the occupied surface area. In this system single spheres were plated in each well of a 96-well plate and the surface area of spheres was measured in two dimensions on day of plating (day zero) and the same spheres were followed for three days measuring the surface area occupied in two dimensions by the migrating cells. the total migrated area on each day was divided by the surface area of the respective spheres on day zero, in order to find the fold change in the occupied surface area. That way, I could normalize the migrated area to the initial size of the sphere which produced more precise

measurements. This method was used repeatedly for most of the GBM cell types we had. However, some GBM lines do not form large enough spheres for reliable measurements of the initial size as well as the migrated area. This caused our experiments to be confined to the GBM lines forming large enough spheres.

Furthermore, EFNA3 knockdown in MSCs did not alter the migration pattern of some GBM lines upon exposure to CM from those MSCs. These GBMs were almost always of mesenchymal subtype. Therefore, the migratory behavior observed in the mesenchymal subtypes of GBMs may not be related to EFNA3 in MSCs. Additionally, classical subtypes did not adhere or migrate upon MSC-CM exposure (only increase in viability). As a result, our experiments regarding the effects of EFNA3 in MSCs on the migration of GBMs were confined to proneural subtype of GBMs only.

Those observations lead to a question about the mechanism of action of EFNA3 in the migration of GBMs and about the subtype dependency of CM-mediated migration of GBMs. We have found evidence supporting the view of direct transfer of EFNA3 mRNA from MSCs to GBMs via exosomes. EFNA3 locus harbors a few transcription start sites which are activated by hypoxia, leading to nonfunctional proteins which are not integrated into plasma membrane. Interestingly, those long noncoding RNAs act as competing partners for MiRNAs which target EFNA3, increasing EFNA3 protein abundance in cells causing metastatic dissemination in breast cancer [Gomez-Maldonado et al., 2014]. So, the EFNA3 mRNA delivered via exosomes might be such noncoding EFNA3 mRNA sequences that cause elevate the translation of endogenous EFNA3 proteins causing such mobilization and metastatic behavior. Those nonfunctional mRNAs may not have been distinguished in RNASeq due to arbitrary sequencing of all RNA content in a given sample leading to annotation of even shorter sequences as the intact RNA. Moreover, delivery of coding EFNA3 mRNA via into the GBM cells via exosomes might act in the same way as long noncoding EFNA3 mRNAs leading to the same outcome.

Alternatively, in each sphere, there might be a balance between EFNA3- and EphA-expressing cells in terms of abundance. Upon translation of EFNA3 in cells receiving exosomes and integration into the plasma membrane, this balance is distorted causing repulsive interactions to increase, leading cells migrating away from each other. It might even be that some cells have expression of both the ligand and the receptor in a certain ratio which is also distorted upon exosome reception. Therefore, the endogenous EFNA3 expression levels in GBMs might be important for the determination of the migratory behavior caused by EFNA3 that is deposited into them via exosomes from MSCs. Proneural GBMs express EFNA3 more than mesenchymal subtype [Lottaz et al., 2010]. Addition of more EFNA3 on the cell membrane in proneural subtypes may shift this balance towards EFNA3 causing repulsion and migration. However, mesenchymal subtype GBMs do not possess significant amounts of EFNA3 on plasma membrane, so addition of EFNA3 might not be enough to drive migration, as I showed that mesenchymal GBM cell migration is unrelated to EFNA3, supporting the hypothesis.

Furthermore, I tested the effects of EFNA3 on GBMs in an independent system by expressing EFNA3 in HEK-293 cells and performing the migration assays for GBMs with CM from those HEK-293 cells overexpressing EFNA3 in the same way as for the MSC-CM. GBMs that were

stimulated with CM from EFNA3-overexpressing HEK293 cells migrated to significantly larger areas compared to those stimulated with CM from HEK293 cells carrying the empty vector. However, this increase was lower than the levels obtained with MSC-CM. This might be due to lower amounts of packaging of EFNA3 mRNA into exosomes. It might even be that the exosomes are loaded with EFNA3 protein, but not the mRNA. Yet, this still proves the fact that EFNA3 is involved in the migratory stimulation of GBMs.

Epithelial to mesenchymal transition (EMT) is one of the major mechanisms of cancer cell metastasis. Although CNS lacks the critical component required for such migration, which is the basement membrane (epithelial structure), the key invasion mechanisms overlap between CNS and peripheral tumors [Kahlert et al., 2013]. However, Vimentin (an intermediate filament required and used as marker for EMT) staining did not show a significant difference between GBMs that are cultivated in the presence of CM and control medium. Supporting this finding, Kahlert et al. demonstrated in 2012 that, migrating GBMs display properties similar to GBM mesenchymal subtype through activation of Wnt/β-catenin signaling, making it untraceable by vimentin (Kahlert et al. 2012). This might mean that GBMs exposed to MSC-CM might gain mesenchymal phenotype (from proneural and classical subtypes into mesenchymal phenotypes) which brings also the migratory phenotype. Additionally, in the RNASeq data, CD44 (a mesenchymal marker) expression was increased in the GBMs which received exosomes compared to controls. Moreover, CD133 and Nestin staining in GBM cells which were exposed to CM were increased compared to controls, suggesting more stem-like characteristics of mesenchymal GBMs consistent with the other studies [Lottaz et al., 2010]. Overall, these observations support that serum-free MSC-CM, and the MSCs in the satellite GBMs, render GBMs more mesenchymal (and stem-like) and cause their migration and invasion.

In light of those findings, it can be concluded that EFNA3 is the main mediator of the migration in proneural GBMs and also causes a mesenchymal shift. Using this information, EFNA3 blockers can be developed and tested for efficacy in vitro and in vivo in terms of the prevention of the migration. Those EFNA3 blockers then can be used to perform a helping treatment in GBMs. MSCs home better the satellite tumors, which are the sources of tumor recurrence and deeper infiltration. Therefore, the EFNA3 blockade can help prevent the more diffuse spreading of the GBMs. When satellites develop into larger tumors, they can be easily resected with no risk of leaving infiltrated cells behind which causes the recurrence. This, altogether, might lead to prolonged survival.

However, a major problem could be the localization and abundance of EFNA3 in the body. Eph-Ephrin signaling is important in developmental processes such as in the formation of axonal tracts. In the adulthood they continue to function as boundaries between different tissues or different parts of a specific organ. They are especially abundant in brain. Ephrin-A3 plays a role in the long-term potentiation formation in the brain as well as neuron-glia interactions (Filosa et al, 2009). Furthermore, Ephrin ligand family members have significant sequential and structural similarities which might cause non-specific targeting by the blocker. Therefore, administration of EFNA3 blockers might interfere with nonspecific targets within the body.

5.9. MSCs did not Alter the Median Survival

In addition to showing that the MSCs home satellite tumors better than the main tumor mass, we tested the effect of MSCs on the overall survival rate. The MSCs were again inoculated along with the GBMs. The GBM we inoculated (classical subtype) responded to CM by a significant increase of viability in vitro. Therefore, it was expected to become more aggressive in the presence of MSCs leading to a shorter median survival. However, the difference between median survival of the mice inoculated with GBM cells along with MSCs were not significantly different from those with GBM cells alone. This might be due to more factors coming into play in vivo from which GBMs receive signals that cannot be controlled. Additionally, it is not certain if MSCs were completely isolated from the blood-borne cues, contrary to the in vitro serum-free conditions, which make them more antitumorigenic (Figure 1). Additionally, it was shown by others [Stoff-Khalili et al., 2007; Mohr et al., 2010] and us that MSCs home satellites better than main tumor mass. As being an early study, we only tested for the survival, rather than infiltration and recurrence, where MSCs have more relevance to the clinical situation. Therefore, removal of the main tumor mass and tracking of a relapse in the presence of MSCs would be a better approach in terms of the effects of MSCs in GBM pathogenesis.

Moreover, MSCs which are infused systematically into the blood stream in vivo have been shown to be gradually decreasing after 24 hours [Saat et al., 2016; Xie et al., 2017] becoming nondetectable after 14 days [Kidd et al., 2009]. In our experimental setting, we inoculated MSCs directly into the brain tissue, close to the tumor xenograft, which may have provided a longer survival of them until they reach the tumor. Yet, this does not ensure sustained effects of MSCs on the tumor. Therefore, a repeated systematic injection is required to achieve a stable quantity of MSCs within the tumor which also would be a less invasive approach.

5.10. Information Transfer Between MSCs and GBMs

In the NGS of exosomes and the GBMs that were stimulated with the exosomes, we have demonstrated the existence of information transfer from MSCs to GBMs. In addition, we proved the transfer of the information exchange in an experimental setting in vitro. In this setting, we used a system where Cre-recombinase fused with ERT2 is expressed in MSCs and a flipped-reporter-GFP is expressed in GBMs. This system provides a tamoxifen-inducible labelling of GBMs allowing detection of interactions at a certain time point. And upon tamoxifen induction, the GBMs receiving Cre-ERT2 from MSCs are permanently labelled with GFP allowing tracking of individual cells receiving signals from MSCs. With this experimental system, the information transfer took place in some 5-8% of the GBMs as the MSC population was not completely selected for the existence of the positive selection marker for puromycin resistance. On the other hand, the message that is transferred from MSCs to GBMs requires being packaged into exosomes. We demonstrated the packaging of EFNA3 and Cre-recombinase packaging was established by others [Ridder et al., 2012]. However, we are not sure about whether mRNA or protein of Cre-ERT2 locus is packaged into exosomes as well as to what extent this packaging takes place in MSCs, contrary to the findings of Ridder et al, who demonstrated the packaging of Cre-ERT2 mRNA into exosomes in cells from hematopoietic

lineage. That's why further optimizations are required to achieve a higher rate of recombination events.

Furthermore, we would like to combine this system with the TK system in a way that one GBM cell will have both systems (TK and Flip-GFP). This way, with GCV, we will annihilate most of the GBM cells (with the TK suicide gene) causing formation of satellite tumors and the MSCs carrying Cre-recombinase insert will home the remaining satellites. Via the information transfer, the GBMs which receive exosomes from MSCs will be marked with GFP permanently. This system, therefore, will allow us to follow the behavior of each GBM which receive exosomes from MSCs in terms of proliferation and migration.

5.11. MSCs Render GBMs Chemoresistant

There is a body of research about MSCs being involved in chemoresistance in tumors (Roodhart et al, 2011; Ji et al, 2016; Maj et al, 2017). So, I also tested the effects of MSCs on GBMs in terms of chemoresistance. Some GBM lines which are killed by TMZ under normal conditions become more resistant and some of them are completely irresponsive to TMZ when they are cultivated in MSC-CM. QKI proved to be important in this phenomenon. As both CM and the QKI overexpression (via increase in EGFR levels) in common mediate proliferation, the chemoresistance effect could be related to a decrease in TMZ/cell ratio as the cells in CM will proliferate faster than those in control medium, allowing GBM cells to escape the cell cycle arrest due to damage to DNA introduced by TMZ. Additionally, via immunocytochemical methods, I found out that MSC-CM increases the stem cell markers, such as CD133 and Nestin as well as the proliferation marker Ki67, in GBMs and decrease the differentiation markers. Moreover, a mesenchymal subtype shift is observed in GBMs upon exposure to MSC-CM (mentioned above). Mesenchymal GBM subtype expresses relatively more stem-cell markers and they are more resistant to genotoxic insults leading to a more aggressive phenotype. These show that the GBMs become more mesenchymal, stem-like and chemoresistant and eventually more aggressive upon contact with MSCs in serum-free conditions.

6. CONCLUSION

In this study, I demonstrated that MSCs home GBMs, especially at the invasive tumor front, i.e. tumor satellites where they are away from bloodstream. In serum-free in vitro conditions mimicking the satellite tumor microenvironment, they act as pro-tumorigenic by elevating proliferation and viability of GBMs via signaling through soluble factors leading to an increase in intracellular QKI levels, thereby de-repressing and increasing EGFR levels. Moreover, via EFNA3 mRNA packaged into exosomes they release, they enhance the migration of GBMs. Furthermore, they render GBMs chemoresistant against the conventional therapeutic agent, TMZ. MSCs cause a mesenchymal shift in the proneural and classical subtype of GBMs leading to more stem-like and invasive phenotype, which, likely, accounts for the significant increase in migration and chemoresistance.

MSCs are more relevant to the relapse of GBMs in the clinical scenario where main tumor is removed leaving deep infiltrative cells and satellites. Thereafter, MSCs cause regrowth and further infiltration of GBMs to the surrounding tissue. In order to study this pathological situation, we developed a tumor satellite model where GBMs expressing TK suicide gene are treated with systematical GCV injection, causing elimination of a majority of GBM cells, leaving small quantities which form satellite tumors. Additionally, we developed a system to test the mRNA transfer from MSCs to GBMs utilizing a Cre-ERT2 fusion gene expression in MSCs and a flipped-GFP expression in GBMs. This system allows for the demonstration of direct message transfer from MSCs to GBMs, for the temporal control over the recombination events as well as for permanent labelling of GBM cells which receive exosomes from MSCs making them trackable throughout the development of the tumor. However, this system requires further optimization in terms of the efficiency and frequency of the transfer and recombination events.

Future studies will utilize the Cre-ERT2 and Flipped-GFP system in combination with the satellite tumor model, in order to investigate the interaction between MSCs and GBMs at satellite tumors, permanently labelling GBM cells receiving exosomes from MSCs so that they could be tracked during their development in terms of migration as MSC exosomes cause migration of GBMs. Furthermore, EFNA3 blockade will be tested for preventing MSC-mediated migration of GBM cells. Overall, these findings shed light on the MSC-GBM interactions further, describe a, to our knowledge, previously unknown EFNA3 transfer, and the developed methods enable us to perform further experimentations more precisely in order to investigate the effects of MSCs on GBMs further.

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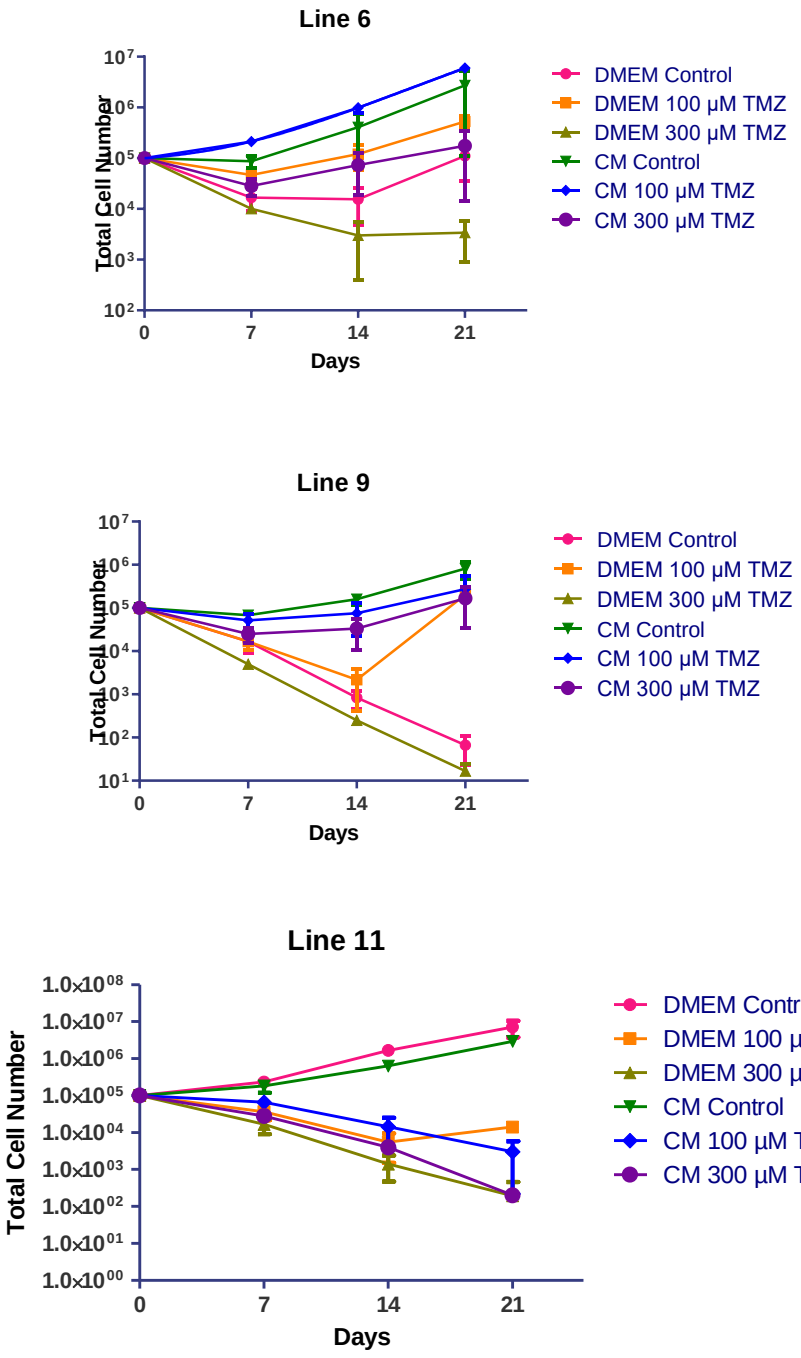
Supplementary Figure



Supplementary Figure 1: CM rescues GBMs from TMZ effects. Additional lines were subjected to TMZ in DMEM or CM from MSCs (serum-free). The calculation of total cell numbers was described above (Figure 8). GBMs treated with TMZ cultivated in CM had larger numbers of cells compared to those cultivated in control (DMEM).