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Expression of Glia Maturation Factor in human glioblastoma cells and its impact on cellular stress response after hypoxia

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Abbreviations

AKT	protein kinase B
APS	Ammonium peroxosulphate
ATF-2	activating transcription factor-2
BSA	Bovine serum albumin
CTRl.	non-transfected control cells
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
e.g.	exempli gratia
EAE	experimental autoimmune encephalitis
et al.	et alii
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal-related kinase
FBS	fetal bovine serum
GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase
GBM	glioblastoma multiforme
GMF	glia maturation factor
GMFsiRNA	small interfering ribonucleic acid targeted against glia maturation factor
Grb-2	Growth-factor-receptor-bound-2
H	Hour
HER	human epidermal growth factor receptor
HIF-1	hypoxia inducible factor-1
Hrs.	hours
i.e.	id est
IDH1	isocitrat dehydrogenase
IL-1	interleukin-1
JNK	c-Jun amino (N)-terminal kinase
kDA	kilo Dalton
LOH	Loss of heterozygosity
MAP	mitogen- activated protein

MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MDM2	mouse double minute 2 homologue
MEK	mitogen- activated protein kinase kinase
MGMT	methyl guanine methyl transferase
MK2	mitogen- activated protein kinase-activated protein kinase-2
mTOR	mammalian target of rapamycin
NfκB	nuclear factor-κB
NGF	nerve growth factor
NsiRNA	nonsense small interfering ribonucleic acid
P14ARF	alternative reading frame product of cyclin-dependent kinase inhibitor 2A
P38	protein 38
P53	protein 53
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PDGFR-a	platelet derived growth factor receptor-a
PI3K	phosphatidylinositol-3-kinase
PIP3	phosphatidylinositol (3, 4, 5)-triphosphate
PKB	protein kinase B
PKC	protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PTEN	phosphatase and tensin homologue
PLC	phospholipase C
RAF	Rapidly growing fibrosarcoma
RAS	Rat sarcoma
SDS	sodium dodecyl sulphate
siRNA	small interfering ribonucleic acid
Sos	Son of sevenless
TEMED	Tetramethylethylendiamine
TNF- α	tumour necrosis factor- α
UV	ultra violet
WHO	World Health Organization

A. Introduction

Today the most frequent cause of death after cardiovascular disease is the death because of cancer disease in Germany. Glioblastoma (GBM), a WHO grade IV astrocytic brain tumour, accounts for about 45% of all primary intracerebral tumours with a median survival of 14.6 months from diagnosis (Stupp et al. 2009). This neoplasm is characterized by deregulation of many signalling pathways involving growth, proliferation, survival and apoptosis which promotes the highly resistant character of tumour cells. The infiltrating growth regularly causes tumour recurrence (Hofer and Herrmann 2001). The multimodal therapy of glioblastoma includes maximal surgical resection, radiotherapy and chemotherapy; however resistance of tumour cells to these treatments and diffusely infiltrating growth keep the results of the therapeutic strategies moderate. Furthermore, recent studies illuminated the importance of the inflammatory and hypoxic environment of brain tumours for their resistance and invasiveness (Kim et al. 2009, Sen 2011).

One brain dominant protein, the Glia Maturation Factor (GMF), plays a crucial role in intracellular stress responses and inflammatory processes in neuronal and glial tissues e.g. in Alzheimer's disease (Thangavel et al. 2012). Preliminary results of our working group (not published) pointed to possible expression of GMF in gliomas. The aim of the present work was to demonstrate the expression of GMF in human GBM cell lines and its impact on central stress signalling pathways.

1. Glioblastoma

1.1. Classification by the WHO of astrocytic tumours

Glioma is a general term to define primary tumours of the central nervous system originating from macro glial cells or their precursors. The main groups of gliomas resemble three different cell types: astrocytomas, oligodendrogliomas and ependymomas; besides there are mixed tumours with different cell types such as oligoastrocytomas. The astrocytomas are the most common gliomas – more than 80% of all malignant primary brain tumours show characteristics

of astrocytes (Dolecek et al. 2012). Normal astrocytes feature supportive functions for neuronal cells and their processes surround the brain capillaries.

Astrocytic tumours are graded according to the World Health Organization (WHO) into grade I – IV by their histological hallmarks including elevated cellularity, cytological atypia, high mitoses activity, endothelial proliferation and necrosis (Louis et al. 2007). WHO grade I tumours retain their well-differentiated features and show circumscribed growth, whereas tumours of grade II-IV tend to grow infiltrating into surrounding brain tissue and are more and more de-differentiated. Malignant gliomas often show different histological characteristics. Low grade tumours like diffuse astrocytoma (WHO II) show only atypical cytological features alone, whereas malignant gliomas like anaplastic astrocytoma (WHO III) and giant cell glioblastoma, gliosarcoma and glioblastoma (WHO IV) show different histological characteristics like anaplasia and elevated mitosis (WHO III) and in addition neovascularization and necrosis (WHO IV) (Louis et al. 2007). The grading correlates negatively with the five years survival rates as shown in Table 1.

Table 1: Grading system of astrocytomas according to "The WHO grading of the central nervous system" and corresponding patient survival rates (Louis et al. 2007, Dolecek et al. 2012).

Grade	Denotation	Histological Criteria	5-year survival rate
I	subependymal giant cell astrocytoma, pilocytic astrozytoma	non-infiltrating, circumscribed growth	80%
II	diffuse astrocytoma, pilomyxoid astrocytoma, pleomorphic xanthoastroytoma	diffusely infiltrating, nuclear atypia	47%
III	anaplastic astrocytoma	diffusely infiltrating, nuclear atypia, increased cellularity, mitotic activity	30%
IV	glioblastoma, giant cell glioblastoma, gliosarkoma	as grade III + nuclear atypia, mitotic activity, endothelial proliferation, tumor necrosis	4%

Accounting for approximately 60-75% of all astrocytic glial tumour types glioblastoma (GBM) is the most common and histopathologically most malignant primary brain tumour in adults (Ohgaki and Kleihues 2005).

Some histological hallmarks of glioblastoma are areas of intratumoural necrosis surrounded by pseudopalisading cells and vascular proliferation. Therefore, presence of hypoxic regions is a typical finding in glioblastoma (Brat et al. 2004).

1.2. Subgroups of Glioblastoma

Over 95% of GBM emerge *de novo* with no pathologic precursor lesion (primary GBM) and 5% are thought to develop through progressive changes from lower grade astrocytomas (secondary GBM). It could be shown that glioblastoma present different genetic alterations which gave reason to classify GBM in primary and secondary Glioblastoma.

GBM morbidity rate peaks among 45-65 years, men are affected about 1.6 fold more often than women (Ohgaki and Kleihues 2012). *De novo* GBM more often infest in older patients with a mean age of 62 years, while secondary GBM mostly affect younger patients.

Mutations of IDH1 (isocitrat dehydrogenase 1) are the earliest detectable genetic alteration in precursor low-grade diffuse astrocytomas and in oligodendroglioma but not in primary GBM.

These findings may indicate that primary and secondary GBM are derived from different precursor cells and present different tumourigenesis. It was concluded that this genetic alteration is a definitive diagnostic molecular marker of secondary glioblastoma (>80%) and more reliable and objective than clinical criteria (Ohgaki and Kleihues 2007).

Furthermore, GBM is characterized by deregulation of many signalling pathways involving growth, proliferation, survival and apoptosis e.g. phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB or AKT), NfκB (nuclear factor- κB), mitogen activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) and phospholipase C (PLC)/ protein kinase C (PKC) pathways. Many genes of these pathways such as *PTEN* (phosphatase and tensin homologue), *EGFR* (epidermal growth factor receptor), *PDGFR-α* (platelet derived growth factor receptor-a), *p53* (protein 53) and *mTOR* (mammalian target of rapamycin) are decontrolled by amplification, mutation or overexpression (Soni et al. 2005, Brennan et al. 2009, Mercer et al. 2009, Roesler et al. 2010). These deregulations cause many features of both primary and

secondary GBM. Glioblastoma gains ability to sustain angiogenesis, to invade normal tissue and to become independent of external growth or apoptotic signals (Hanahan and Weinberg 2000).

A key signalling pathway of *de novo* GBM is the EGFR/PTEN/AKT/mTOR pathway. In normal cells activated EGFR leads to many different cell responses such as migration and differentiation as well as homeostatic functions. Amplifications of the *EGFR* gene are observed in more than 40% of primary GBM but rarely in secondary GBM (Ohgaki et al. 2004). EGFR amplification leads to overexpression and is associated with constitutive, i.e. permanent activation of the receptor. Activation of EGFR can enhance cell survival through activation of phosphatidylinositol (3, 4, 5)-triphosphate (PIP3)/AKT pathway.

The *PTEN* gene (phosphatase and tensin homologue) inhibits the PIP3 signal and is thereby inhibiting proliferation. Loss of PTEN activation results in attenuated inhibition of proliferative pathways with following aberrant proliferation. Loss of PTEN is almost exclusively found in primary glioblastoma (15-40%) (Ohgaki and Kleihues 2007).

Loss of heterozygosity (LOH) 10q is the most frequent genetic alteration of primary and secondary glioblastoma (60-80%). LOH10q is associated with acquiring the glioblastoma phenotype during astrocytoma progression (Fujisawa et al. 1999, Fujisawa et al. 2000). In contrast mutation of p53 plays a crucial role in the development of secondary glioblastoma. This mutation is one of the first detectable alterations in precursor lower-grade astrocytic lesions and is a genetic hallmark of secondary GBM (65% (Louis et al. 2007)).

MDM2 (mouse double minute 2 homologue) inhibits p53 functions and overexpression is observed mainly in primary GBM (over 50%) [11]. p14ARF (an alternative reading frame product of cyclin-dependent kinase inhibitor 2A) inhibits MDM2-mediated p53 degradation, but there was no significant difference in the overall frequency of p14ARF alteration between *de novo* and secondary GBM (Nakamura et al. 2001).

The most frequent markers of the different glial tumours taken together by Ohgaki and Kleihues 2009 are shown in Figure 1.

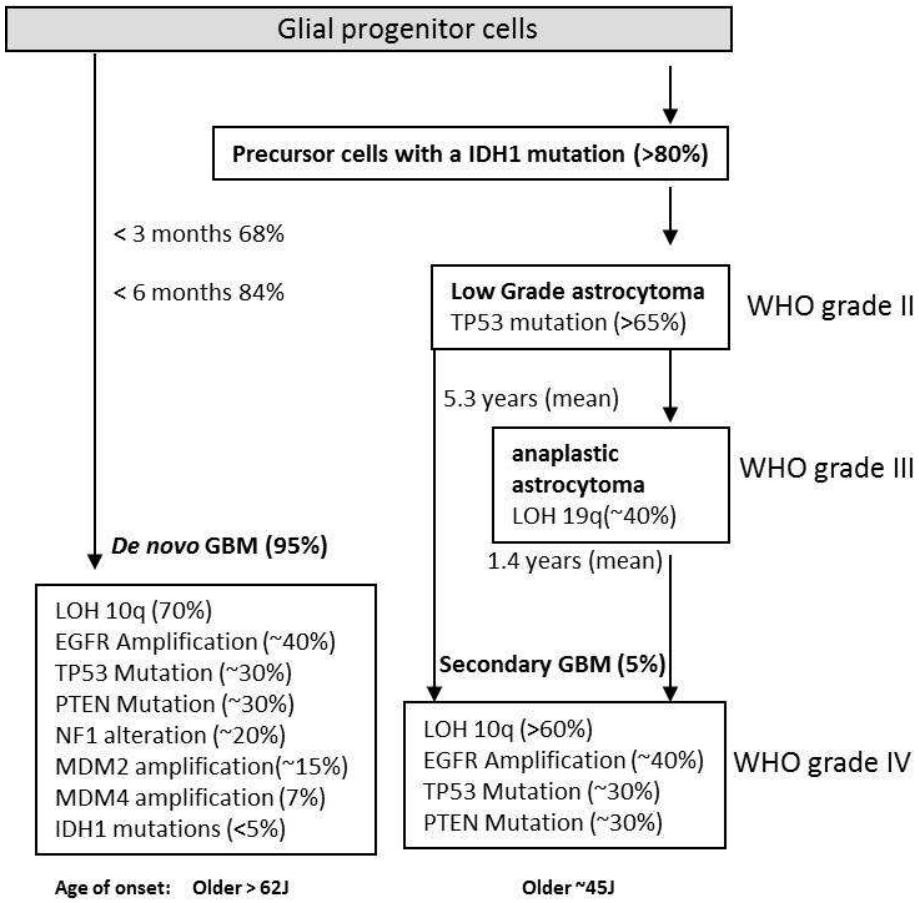


Figure 1: Modified from Ohgaki et al., 2005 and 2009

Understanding of these biological features of glioblastoma should provide the basis for drug discovery and development of new therapy protocols.

1.3. Therapy and Prognosis

Standard therapy of glioblastoma currently consists of gross total resection, combined radiation therapy and chemotherapy and adjuvant chemotherapy. Addition of Temozolomide, a DNA alkylating agent, to surgery and adjuvant radiotherapy could improve the overall survival of patients with glioblastoma to 14, 6 months. It could be shown that patients with a methylation of methyl guanine methyl transferase (MGMT) - a DNA repair enzyme- had profit from addition of temozolomide to therapeutic surgery and radiotherapy (Marsh et al. 2013). However resistance of tumour cells to these treatments and diffusely infiltrating growth keep the results of these therapeutic strategies moderate.

GBM are considerably heterogeneous, and the intratumoural diversity probable causes therapeutic resistance targeting e.g. the EGFR. Besides the highly resistance rates of this tumour are thought to be due to the tumour microenvironment consistent of inflammation and hypoxia and the existence of highly resistant cells and stem cells (Taylor et al. 2012).

Until today, the clinical outcome could not be improved considerably: a majority of patients have a median survival of 14.6 months from diagnoses (Stupp et al. 2009). There is a high interest in finding more tumour-specific and efficient therapeutic alternatives. Therapy with tumour-specific antibodies and immune-modulating agents are object of many clinical trials and make closer examinations of tumour biology and genetic characteristics necessary.

2. Relevant Pathways

2.1. Glia maturation factor

One brain-dominant protein involved in the MAP Kinases pathways is the Glia Maturation Factor (GMF). GMF (17 kDA) was first purified, sequenced and cloned 23 years ago. It is highly conserved between the species and physiologically localized in astrocytes and in some neurons (Wang et al. 1992).

In vitro GMF is a potent inhibitor of ERK1/2 MAP Kinase and at the same time an enhancer of p38 MAP Kinase (Lim and Zaheer 1996, Zaheer and Lim 1996). The group of Zaheer could show that GMF is an upstream stimulator of p38 MAPK in glioma cells and that transient overexpression of GMF in PC12 pheochromocytoma cells activates p38 MAPK and MK2 (MapKap Kinase-2) (Zaheer and Lim 1998, Lim et al. 2000). In C6 cells as well as in normal astrocytes overexpression of GMF leads to activation of p38 and of NfκB. Recent work demonstrates the prominent expression of GMF in neuropathological lesions of Alzheimer's disease (Thangavel et al. 2012) and in inflammatory models like experimental autoimmune encephalitis (EAE) (Zaheer et al. 2007). Kaimori et al. could show that overexpression of GMF in renal tubular cells makes them susceptible to oxidative stress by apoptotic mechanism (Kaimori et al. 2003).

In serous ovarian cancer, GMF expression was significantly enhanced when compared to normal epithelium. In these tumours a strong correlation between overexpression of GMF and clinical pathological features of cancer patients like diminished disease-free survival and less overall survival could be demonstrated (Li et al. 2010). Accordingly GMF seems to play an important role in promoting inflammatory cell responses via p38 pathway; beside this GMF overexpression leads to vulnerability to oxidative injury through the p38 pathway (Li et al. 2010).

These findings suggest an important role of GMF in the regulation of intracellular signal transduction after stress stimuli.

2.2. MAP Kinases

Mitogen-activated protein kinases (MAPKs) are components of a complex intracellular signalling network. Because of their importance during developmental processes and the survival or death responses of cells, the MAPK pathways have become the focus of attention in recent years.

Mitogen-activated protein kinases are protein kinases that convert extracellular stimuli into diverse intracellular responses. Most extensively studied groups are the c-Jun amino (N)-terminal kinase (JNK), the p38 isoforms and extracellular signal-regulated kinases 1/2 (ERK1/2). Specific stimuli activate mitogen-activated protein kinase kinase kinases (MAPKKKs) which then phosphorylate different isoforms of mitogen-activated kinase kinases (MAPKKs or MEK). These MAPKKs in turn activate the MAPKs p38, JNK and ERK1/2.

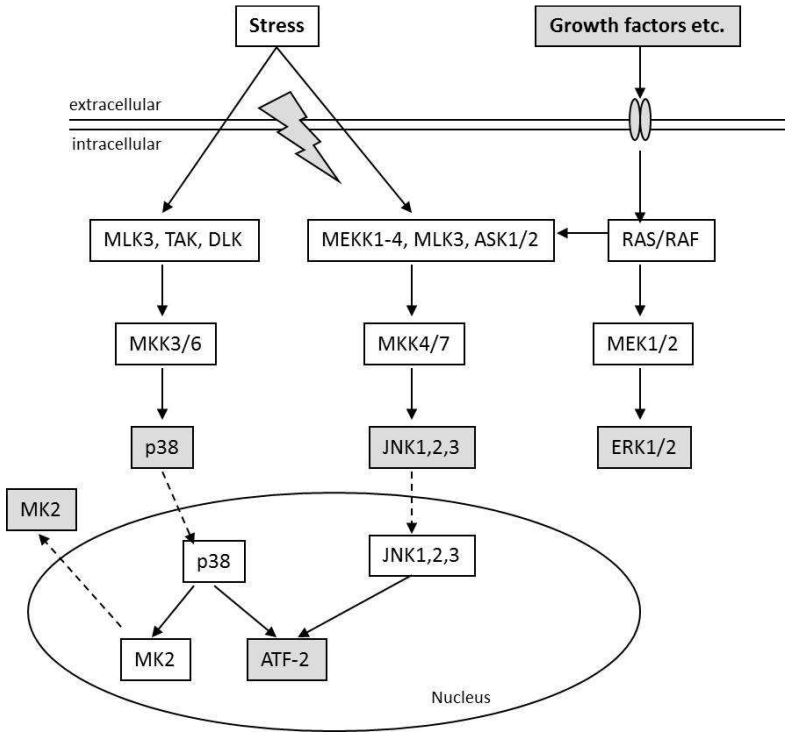


Figure 2: Overview over MAP Kinase pathways. Potential cross talk or other substrates are left out for clarity. Modified from cellsignal.com

2.3. p38 MAP Kinase

Similar to JNK the p38 MAPK is strongly activated by cytokines and exposure to environmental stress. The major protein kinases responsible for activation of p38 are thought to be MKK3/6, and subsequently upon stimulation p38 isoforms phosphorylate different substrates in many cellular compartments.

p38 MAPK plays a critical role in inflammatory cell response, cell proliferation and survival inducing apoptosis after cellular stress (Cuenda and Rousseau 2007, Cuadrado and Nebreda 2010). In these biological processes dependent on p38 the MAPKAP kinase-2 (MK2) plays a role for cellular response to external stress stimuli e.g. for actins remodelling and cell migration, cytokine production, transcriptional regulation and cell cycle control (Cargnello and Roux 2011). One substrate of p38 performing its function in the nucleus is the activating transcription factor-2 (ATF-2) which is activated by phosphorylation upon stress stimuli.

Demuth et al. observed that p38 is a strong promoter of tumour invasion, progression, and poor patient survival. Inhibition of p38 leaded to significantly reduced glioma invasiveness in vitro (Demuth et al. 2007).

2.4. c-Jun amino (N)-terminal kinase (JNK)

The c-Jun NH2-terminal kinase (JNK) represents one subgroup of MAP kinases. JNK is activated mainly by various environmental stresses and inflammatory cytokines including oxidative stress, UV irradiation, hypoxia, ischemia, interleukin-1 (IL-1) and tumour necrosis factor α (TNF-α). Although former studies widely illuminated the importance of the JNK pathway cascade for neuronal cell death, other works have it implicated in both apoptosis and survival signalling depending on cell type (reviewed in (Liu and Lin 2005)).

It has been shown that constitutive activation of JNK correlates with histological grade and EGFR expression of diffuse gliomas (Li et al. 2008). Inhibition of JNK and subsequent inhibition of phosphorylation of c-Jun leads to enhanced temozolomide- induced cytotoxicity in human glioma cells (Ohba et al. 2009).

2.5. MAPKAP 42/44 or ERK1/2

In contrast ERK1/2 is activated by MEK1/2 after stimulation by mitogen agents and growth factors like platelet-derived growth factor (PDGF), epidermal growth factor (EGF), nerve growth factor (NGF) and insulin. It plays an important role in the control of cell proliferation and survival. Previous reports suggest that the ERK pathway is activated in tumour areas exposed to hypoxia and that activation of ERK 1/2 is involved in proliferation of U87MG cells under hypoxia (Fang et al. 2010, Zhang et al. 2012).

2.6. Epidermal growth factor

The epidermal growth factor receptor (EGFR) belongs to the ErB/HER family of tyrosine kinases. It is a membrane-associated receptor and ligand binding induces activation of the intrinsic kinase domain. Physiologically, EGFR is responsible for most of the pleomorphic cell responses which include cell proliferation, migration, and differentiation as well as homeostatic functions. Activated EGFR stimulates a wide range of intracellular pathways, which furthermore show

extensive overlap (Yarden and Sliwkowski 2001). In glioma cells under normoxic conditions, EGFR signalling may enhance proliferation, invasion and migration (Lund-Johansen et al. 1990).

One of the most commonly encountered abnormality in glioblastoma is the deregulation of the epidermal growth factor receptor (McLendon et al. 2007). Amplification and overexpression are observed in the majority of *de novo* (primary) glioblastoma and associated with worse prognosis (Shinojima et al. 2003, Ohgaki and Kleihues 2007). Two important pathways downstream of EGFR which transfer the signal on proliferation and cell survival are the PI3K /AKT/mTor pathway and the MAP Kinase/ERK pathway (see below).

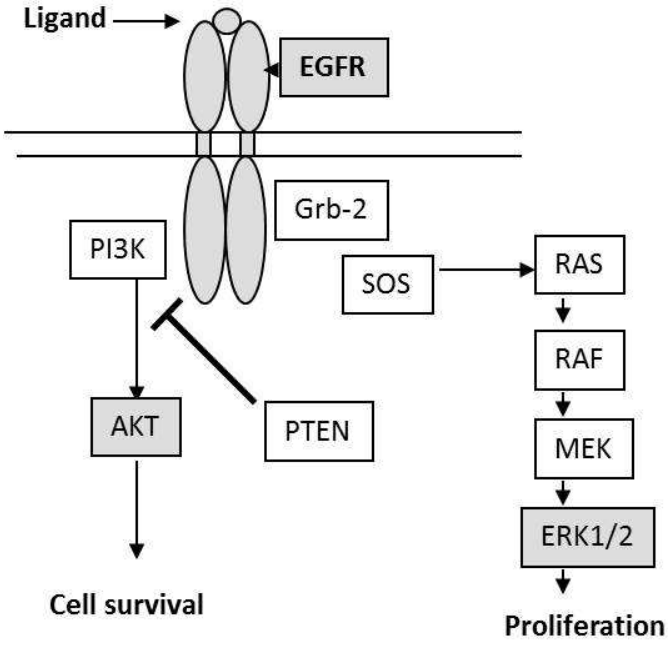


Figure 3: Two main pathways which are transferring the signalling cascades for cell survival or proliferation downstream to EGFR. Potential crosstalk is left out for clarity.

AKT is a serine/threonine kinase and an important node of the PI3K/AKT pathway. PI3K activation phosphorylates PIP3; AKT is the principal target of PIP3. Once activated, AKT phosphorylates many different target proteins which regulate various cellular functions like angiogenesis, cell survival/escape of apoptosis and cell cycle progression (reviewed in (Lino and Merlo 2011)). A significant correlation between activated AKT and EGFR status, tumour grade and survival could be shown (Osaki et al. 2004, Mizoguchi et al. 2006).

PTEN tumour suppressor gene negatively regulates the activity of PI3K. Loss of function mutations of *PTEN* are frequent in GBM and activate AKT in a similar way as gain of function mutations upstream of AKT. Epigenetic and genetic inactivation of PTEN is associated with shorter survival of patients with GBM (Lino and Merlo 2011).

3. Hypoxia and glioblastoma

GBM is characterized by invasiveness, necrosis and angiogenesis. In particular, vascular proliferation is an important aspect of GBM and correlates with the grade and aggressiveness of the tumour (Borovski et al. 2009, Takeuchi et al. 2010). The increased vascular proliferation is thought to depend on hypoxic conditions created by the elevated growth rate of GBM. Increasing tumour size requires that GBM tumour cells maintain a balance between adaptation to hypoxia and cell death (apoptosis and central necrosis) through the activation of transcription of over 100 different genes.

A major mechanism mediating adaptive responses to hypoxia is the regulation of HIF-1 (hypoxia inducible factor-1). HIF-1 is a heterodimeric protein that is composed of a constitutive expressed HIF-1β subunit and an O_2-regulated HIF-1α subunit. During hypoxia, HIF-1α is stabilized by dimerization with HIF-1β and regulates various genes such as those involved in angiogenesis or transport of oxygen. Moreover those protein products increase oxygen availability or help adapt to insufficient levels of oxygen. The stabilization of HIF-1α is a hallmark of hypoxia, therefore detecting HIF-1α is routinely used to screen hypoxia. In the majority of primary human cancers immunohistochemical analysis revealed increased level of HIF-1α (Semenza 2010). Hypoxia is supposed to play an important role in the characteristics of tumourigenesis including malignant progression. Several works showed an association of hypoxia and e.g. proceeding metastasis, GBM cell migration, tumour recurrence or resistance to chemotherapy and radiation therapy (Gatenby et al. 1988, Hockel et al. 1998). There is a close association of hypoxia and progression of glioma: in fact the degree of necrosis within glioblastoma correlates inversely with patients' outcome and survival (Jensen 2006). There seems to be an association of volume and intensity of hypoxia and the time to tumour progression and survival (Spence et al. 2008). In addition

recent works showed that hypoxia may be a critical factor for the microenvironment for cancer stem cells and neuronal stem cell tropism (Zhao et al. 2008).

For this reason understanding the regulations of signalling pathways in GBM under hypoxic conditions as a target is an attractive therapeutic approach for GBM.

4. Aim of the study

Glioblastoma is one of the highest lethal human neoplasms, and till today it resists to current therapeutic strategies. Therefore, a high scientific interest exists to analyse the molecular pathways involved in therapy resistance in order to define targets for innovative therapeutic strategies.

GMF is a peptide molecule involved in intracellular signal transduction pathways. It has been shown to be functional in inflammatory brain tissue as shown for Alzheimer's disease. Preliminary unpublished results of our working group referred to differentially expressed GMF mRNA in glioma cells.

Therefore, the aim of the present study was to investigate the expression of GMF in human GBM and to further analyse the characteristics of its functional role. Thus, GMF was investigated at the protein level in different glioma cell lines. Recombinant downregulation by siRNA technologies was achieved. Using this approach the response of central stress pathways in cells with downregulated GMF and in control cells were investigated under normoxic and hypoxic conditions.

B. Materials and methods

1. Technical Devices

Device	Model	Producer
Centrifuges	5471R	Eppendorf AG, Hamburg, Germany
CO_2incubator	HERAcell®	Thermo Fisher Scientific Inc., Waltham, MA, USA
Electrophoresis cell	Mini-Protean®tetra cell	Bio-Rad Laboratories, Inc., Munich, Germany
Microscopes	Axiovert 25	Carl Zeiss AG, Jena, Germany
pH-meter	EL-30	Mettler-Toledo GmbH Giessen, Germany
Power supplies	PowerPac 300 PowerPAC HC	Bio-Rad Laboratories, Inc., Munich, Germany
Semi-dry transfer cell	Trans-Blot®SD	Bio-Rad Laboratories, Inc., Munich, Germany
Shaker	Minishaker MS1 Orbital Shaker IKA-VIBRAX-VXR	IKA®-Werke GmbH & Co.KG, Staufen, Germany

2. Chemicals and reagents

Substances	Abbreviations	Producer
Acetic acid		Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Amino-n-caproic-acid		Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Ammonium peroxosulphate	APS	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Bovine serum albumin	BSA	Bio-Rad Laboratories, Munich, Germany
Bromphenol-blue		Sigma-Aldrich, Munich, Germany
Dimethylsulfoxide	DMSO	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Dithiothreitol	DTT	Sigma-Aldrich, Munich, Germany
Ethanol		Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Glycerol		Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Glycine		Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Methanol		Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Phenylmethylsulfonyl fluoride	PMSF	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Phosphate buffered saline	PBS	PAA, Pasching, Austria
Rotiphorese NF-acrylamide/ bis-acrylamide solution		Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Skimmed milk powder		Merck KGaA, Darmstadt, Germany
Sodium dodecyl sulphate	SDS	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Tetramethylethylendiamine	TEMED	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Tris		Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Triton-X-100		Sigma-Aldrich, Munich, Germany
Tween-20		Carl Roth GmbH + Co.KG, Karlsruhe, Germany

3. Software

Software	Software producer
Adobe®Photoshop®CS5, Adobe®	Adobe System incorporated, San Jose, CA, USA
Scion Image®	Scion Image®

4. Cell Culture

4.1. Media and additives, consumables

Substances/Materials	Abbreviations	Producer
Accutase		PAA, Pasching, Australia
Dulbecco`s Modified Eagle Medium	DMEM	Life Technologies, Darmstadt
Fetal bovine Serum	FBS	Biochrom AG, Berlin, Germany
Penicillin /Streptomycin		Biochrom AG, Berlin, Germany
Phosphate buffered saline	PBS	PAA, Pasching, Australia
Puromycindihydrochloride	Puromycin	Sigma-Aldrich, Munich, Germany
Serological pipettes, cell scraper		SARSTEDT AG & Co., Nümbrecht, Germany
Centrifuge tubes		
Tissue culture dishes/flasks/test plates		TPP, Trasadingen, Switzerland

4.2. Buffers and media

Cell freezing medium	90%FBS, 10%DMSO
DMEM + FBS	DMEM supplemented with 10% (v/v) FBS, 2m glutamine, 100 U/ml penicillin and 100µg/ml streptomycin

4.3. Cultivation

The human cell line LN18 was provided by Division of Neuropathology, Institute of Pathology, Technische Universität München, Ismaninger Str.22, 81675 Munich, Germany.

Authentication was performed prior to the experiments by analysing microsatellites with Cell IDTMSystems (Promega Corporation, Madison, USA) and tested by a nested PCR for Mycoplasma infections. The human glioblastoma cell line LN18 grows in a bipolar or stellate shape and shows polymorphic nuclei. It was shown that LN18 synthesizes large quantities of fibronectin, in contrast the synthesis of glial fibrillary acidic and S-100 proteins could not be demonstrated (Ishii et al. 1999).

Cells were maintained in DMEM + FBS under standard cell culture conditions at 37°C/ 5%CO2 and passaged twice weekly. Therefore, 1,5ml accutase was applied per 10cm-diameter culture dish for cell detachment.

4.4. GMF knockdown with siRNA

Small interfering RNAs (siRNAs) are short double-stranded nucleic acids, commonly containing 19–21 residues and 3'-dinucleotide overhangs, which are widely used as synthetic reagents to reduce gene expression of target RNA in cells (Jones et al. 2004).

To perform knockdown of GMF gene, cells were transiently transfected with siRNA targeted against Glia maturation factor (GMFsiRNA).

For siRNA experiments, 4.5 x 10^5 cells/well were plated on 6 well-plates 24 h prior to siRNA treatment and incubated. At a sub-confluent level, fresh medium was added prior to transfection. Transfection with siRNA targeted against Glia maturation factor (GMFsiRNA) was carried out using Lipofectamine and OPTI-MEM Reduced Serum Medium essentially as recommended by manufacturer (Life Technologies, Grand Island, NY, USA). Cells transfected with nonsense siRNA (NsiRNA) (Cell Signaling Technology, Danvers, United States) as well as non-transfected cells (CTRl.) served as negative control. After 24h medium was changed to fresh medium and cells could recover for 48h.

4.5. Hypoxia

Cells act more naturally in an environment that is as close to physiological as possible. Most cells exist in environments containing 2 % to 8 % dissolved oxygen - significantly lower than the concentration found in our atmosphere (about 21 %). Therefore low hypoxic conditions should be achieved by an oxygen concentration below 1 % dissolved Oxygen.

To achieve hypoxic conditions, LN18 glioblastoma cells were plated in 6 well-plates and cultured like described above. After recover time wells were placed into air-tight aluminium chambers at 37°C and connected to a vacuum pump and a gas cylinder containing a mixture of 95% N_2 and 5%CO_2. By alternating 11 times between oxygen evacuation and N_2 inflow, an oxygen concentration below 0.66 % (5 mm Hg) was reached in the medium after 22 min.

It could be shown in different cell lines that diverse gene expression programs depend on the time of subjecting the cells under hypoxia (Chi et al. 2006). For that reason LN18 cells were kept under hypoxic conditions for 30 min, 1 h and 6 hrs. and were then extracted.

For normoxic conditions cells transfected with GMFsiRNA, NsiRNA and CTRl. were kept in the incubator and extracted after 30 min, 1 or 6 hrs.

5. Western Blot analysis

5.1. Buffers and solutions

5 x SDS protein sample buffer	312.5 nM Tris-Cl pH 6.8, 10 %(w/v) SDS, 50 %(v/v) glycerol, 250 mM DTT, 0.05 % (w/v) Bromphenol-blue, ad 10 ml H_2O
10 x SDS running buffer	250 mM Tris, 1.92 M glycine, 1% (w/v) SDS ad 500 ml H_2O
10 x TBS buffer	24.2 g Tris, 80.0 g NaCl, pH 7.6 with acetic acid, ad 1000 ml H_2O
Blocking buffer	1 x TTBS + 5 % (w/v) skimmed milk powder
Membrane stripping solution	100 ml methanol, 100 ml acetic acid, ad 1000 ml H_2O
Primary antibody solution	1 x TTBS
Secondary antibody solution	1 x TTBS + 5 % (w/v) skimmed milk powder

Transfer buffers:	
Anode I	300 mM Tris, 20 % (v/v) methanol, ad 500 ml H_2O
Anode II	25 mM Tris, 20 % (v/v) methanol, ad 500 ml H_2O
Cathode	25 mM Tris, 20 % (v/v) methanol, 40 mM amino-n-caproic-acid ad 500 ml H_2O
Washing buffer (TTBS)	1 x TBS, 0.1 % (v/v) Tween-20

5.2. Antibodies

Antibody	Dilution	Producer
Anti-mouse HRP-linked pAb	1:10000	New England Biolabs, Frankfurt, Germany
Anti-rabbit HRP-linked pAb	1:10000	New England Biolabs, Frankfurt, Germany
ERK1/2 rabbit pAb	1:2000	New England Biolabs, Frankfurt, Germany
GAPDH mouse mAb	1:50000	Sigma Aldrich, Munich, Germany
GMF rabbit pAb	1:2000	Sigma Aldrich, Munich, Germany
JNK rabbit pAb	1:1000	New England Biolabs, Frankfurt, Germany
MK2 rabbit pAb	1:1000	New England Biolabs, Frankfurt, Germany
p38 rabbit pAb	1:2000	New England Biolabs, Frankfurt, Germany
phospho-ATF-2 rabbit mAb	1:1000	New England Biolabs, Frankfurt, Germany
phospho-ERK1/2 rabbit mAb	1:1000	New England Biolabs, Frankfurt, Germany
phospho-JNK	1:2500	New England Biolabs, Frankfurt, Germany
phospho-p38 rabbit mAb	1:1000	Millipore, Billerica, USA

5.3. Protein extraction

For cell lysis, 10 x lysis buffer (New England Biolabs) was diluted in H_2O and supplemented with 1 mM PMSF and 1 mM PhosSTOP Phosphatase Inhibitor Cocktail (Roche Diagnostics). Phosphoprotein activation states can act as on/off switches for key cell functions like signal transduction, cell division, cell death and calcium signalling. In order to study these types of factors, endogenous phosphatases must be inactivated to prevent their uncontrolled activity

after cell lysis. These enzymes would otherwise affect the proteins of interest and their activation states. By adding the phosphatase-inhibitor during protein extraction dephosphorylation could be avoided. Adherent cells were harvested with a cell scraper after rinsing with PBS, suspension cells were collected by centrifugation and washed. Following 5 minutes incubation with lysis buffer on ice, cell debris was pelleted by centrifugation at 10000 rpm for 15 minutes. The supernatant was transferred to a fresh tube and amounts of protein were detected by Bradford assay. Binding from coomassie® brilliant blue G-250 (Bio-Rad Laboratories, Munich, Germany) on protein shifts the maximal absorption from 465 nm to 595 nm and quantifies the concentration of protein. Each cuvette was filled with 198 µl PBS, 200 µl Triton-X-100 (0.01% in PBS), 2 µl protein sample and 600 µl Bradford reagent (1:4 in PBS). Absorption was measured at 595 nm with UV/Vis spectrophotometer. For quantification, a calibration-curve with BSA was established (0, 2, 4, 6, 8 µg).

5.4. SDS-PAGE and Western blotting

Equal amounts of protein samples (10 µg) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS in gels binds to and unfolds the protein, giving a near uniform negative charge along the length of the polypeptide. Disulfide bonds are reduced by DTT. Heating the samples at 96°C for 5 minutes with SDS sample buffer promotes protein denaturation and facilitates SDS binding. The protein mixtures were then separated according to their molecular weight in a discontinuous “Laemmli” system. The stacking gel (5%) contains tris-glycine at pH 6.8 and the running gel (10%) resolves at pH 8.8. PageRulerTm plus Prestained Protein Ladder (Fermentas GmbH, St. Leon-Rot, Germany) was as molecular weight marker. Gels for PAGE were either purchased (Mini-PROTEAN TGX Precast Gels; Bio Rad Laboratories) or prepared as follows:

	Stacking gel	Running gel
Gel density	5 %	10 %
Acrylamide [µl]	836	2660
1 M Tris-Cl [µl]	626	2000
Aqua dest [µl]	3500	3320
20% SDS [µl]	25	50
10% APS	25	50
TEMED [µl]	10	25

Acrylamide gels build up cross-links of different sizes dependent on the concentration, the polymerization starts by adding TEMED.

Separation was done at 200 V for 45 minutes with precast gels and at 110 V for 1-2 hours with prepared gels. Afterwards, proteins were transferred to a methanol-activated PVDF membrane (Immobilon-P membrane; Millipore, Billerica, USA) in a semi dry blot system at 225 mA for 50-70 minutes. Blocking of unspecific binding sites was done using 5% skimmed milk powder in TTBS for 1 hour. Protein blots were probed with specific primary antibodies diluted in TTBS overnight at 4°C. After washing 3 x 7 minutes in TTBS the membranes were developed by using the appropriate secondary antibody conjugated to horseradish peroxidase (for one hour at room temperature diluted in TTBS) and by using the Immobilon Western Chemiluminescent HRP Substrate as recommended by manufacture (Millipore, Billerica, USA). Exposures to high-performance chemiluminescence detection film (Amersham Hyperfilm™ ECL™; GE Healthcare Europe GmbH, Munich, Germany) show the immunoreactivity. The intensity of immunoreactivity was measured by densitometry analyses using Scion Image software. GAPDH immunoblots served as protein loading controls.

C. Results

1. Expression of GMF in different GBM cell lines

The four cell lines LN18, LN229, LNZ308 and G139 were analysed by Western Blotting for GMF protein levels. Since the highest expression of GMF was found in LN18, the well characterized cell line LN18 was applied for further experiments.

Figure 4: Expression level of GMF protein in the four Cell-Lines LN18, LN229, LNZ308 and G139

2. SiRNA-mediated downregulation of GMF under normoxic and short time hypoxic conditions

For the assessment of the impact of GMF on downstream signalling pathways in LN18 cells, GMF was downregulated by incubation with 150 nM GMFsiRNA. Following the indicated incubation time protein extraction and Western Blot analyses were performed. GMF was effectively downregulated on protein level (>90 % knockdown) after treatment with siRNA targeted against GMF.

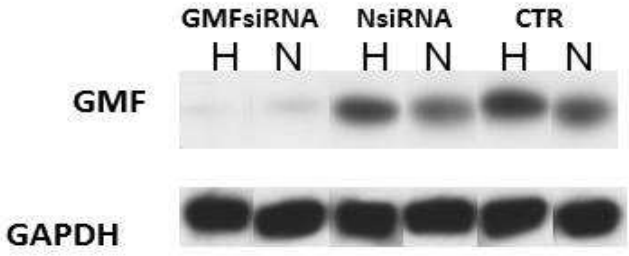


Figure 5: Analysis of GMF protein shows effective downregulation by transfection with GMFsiRNA. After exposure to hypoxia (H) for 1h there is no relevant difference in protein level to normoxia (N). GAPDH serves as protein loading control.

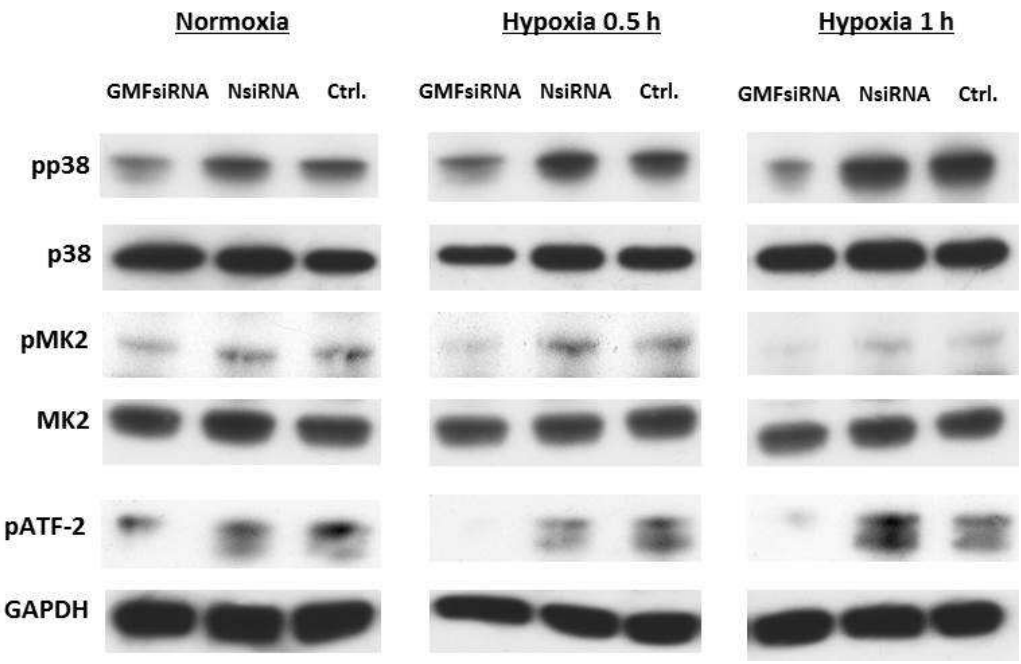
Cells transfected with nonsense siRNA (NsiRNA) and control cells which were not subjected transfection (CTRl.) showed no downregulation. After effective downregulation cells were submitted to hypoxia of about 0, 66% O_2 for one hour. Exposure to hypoxia for one hour did not change protein amount of GMF in LN18 cells.

3. Activation of MAP kinase p38 and downstream substrates ATF-2 and MK2 after downregulation of GMF under normoxic and short time hypoxic conditions

With the background of the implication of GMF on MAPK pathway, p38 phosphorylation e.g. activation as well as the phosphorylation of different substrates of p38 MAP kinase such as MK2 and ATF-2 (activating transcription factor-2) were analysed by Western Blot analyse (Zarubin and Han 2005).

Under normoxic conditions, LN18 cells showed constitutive phosphorylation of p38, ATF-2 and slightly of MK2. Under treatment with GMFsiRNA, phosphorylation of p38 (51% less), MK2 (34% less) and ATF-2 (61% less) strongly decreased. This attenuated phosphorylation under normoxic conditions hold up till one hour of hypoxia. Interestingly, in cells transfected with NsiRNA and in control cells the phosphorylation of p38 and ATF-2 increased under hypoxia (up to one hour) to over 250 % and 300 % in a time dependent manner. In contrast the phosphorylation of MK2 decreased and after one hour under hypoxic conditions there was no phosphorylated MK2 detectable any more.

a)


Normoxia
Hypoxia 0.5 h
Hypoxia 1 h
GMFsiRNA NsiRNA Ctrl.
GMFsiRNA NsiRNA Ctrl.
GMFsiRNA NsiRNA Ctrl.
pp38
p38
pMK2
MK2
pATF-2
GAPDH

b)

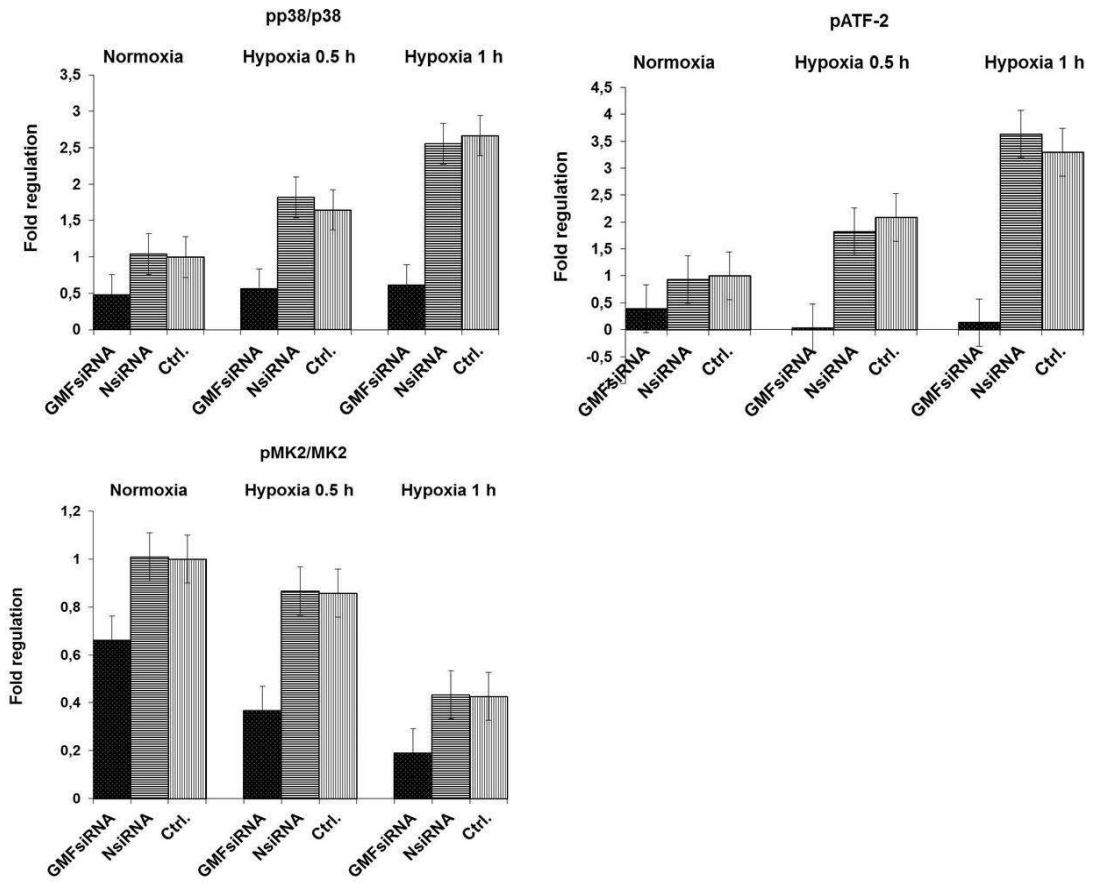
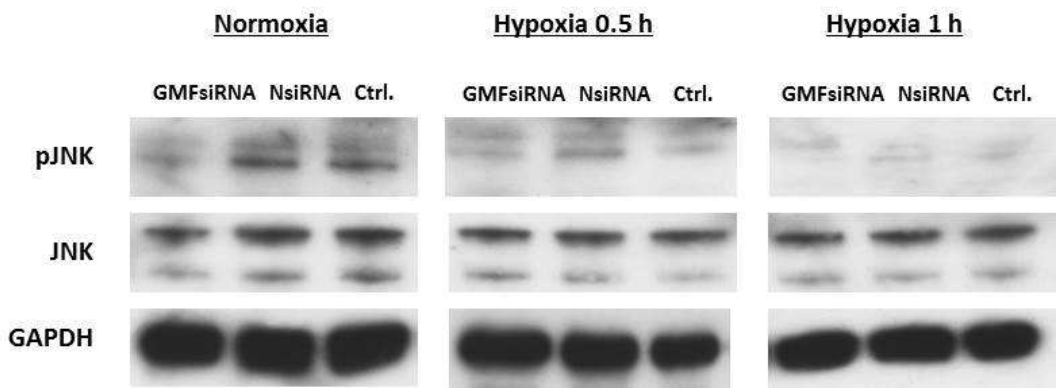

pp38/p38
Normoxia
Hypoxia 0.5 h
Hypoxia 1 h
Fold regulation
3,5
3
2,5
2
1,5
1
0,5
0
GMFsiRNA NsiRNA Ctrl. GMFsiRNA NsiRNA Ctrl. GMFsiRNA NsiRNA Ctrl.
pATF-2
Normoxia
Hypoxia 0.5 h
Hypoxia 1 h
Fold regulation
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
-0,5
GMFsiRNA NsiRNA Ctrl. GMFsiRNA NsiRNA Ctrl. GMFsiRNA NsiRNA Ctrl.
pMK2/MK2
Normoxia
Hypoxia 0.5 h
Hypoxia 1 h
Fold regulation
1,2
1
0,8
0,6
0,4
0,2
0
GMFsiRNA NsiRNA Ctrl. GMFsiRNA NsiRNA Ctrl. GMFsiRNA NsiRNA Ctrl.

Figure 6:
a) Phosphorylation under normoxia and after exposure to hypoxia (0.5 h and 1 h) in cells transfected with GMFsiRNA, NsiRNA and control cells (CTRl.). The phosphorylated protein amount as well as total protein amount is shown above by Western Blot analysis. GAPDH serves as loading control. Under normoxic conditions phosphorylation of p38 and ATF-2 is blocked under treatment with GMFsiRNA, keeping this lower phosphorylated protein level until 1 h under hypoxic conditions. The phosphorylation in cells transfected with NsiRNA and control cells increases until 1 h under hypoxia. PMK2 protein amount decreases in cells transfected with GMFsiRNA but at the same time in contrast to pp38 and pATF-2 pMK2 shows decreasing level of protein in all cells after hypoxic treatment. For AFT-2 I could not establish a valid antibody for the whole protein.
b) Fold regulation of pp38/p38, pMK2/MK2 and pATF-2.

4. Activation of MAP kinase JNK after downregulation of GMF under normoxic and short time hypoxic conditions

Activation of the MAP kinase JNK (pJNK) was detected by Western Blotting. As observed for p38, JNK was phosphorylated in control cells under normoxic conditions. In cells transfected with GMFsiRNA phosphorylation of JNK was attenuated only under normoxic conditions. Following exposure to hypoxia for 0.5 h and 1 h almost no phosphorylation of JNK was observed any more in cells transfected cells with GMFsiRNA, NsiRNA and control cells (CTRl.). They all show nearly the same low phosphorylation of JNK. To sum up after exposure to hypoxia phosphorylation of JNK decreased in a time dependent manner.

a)



b)

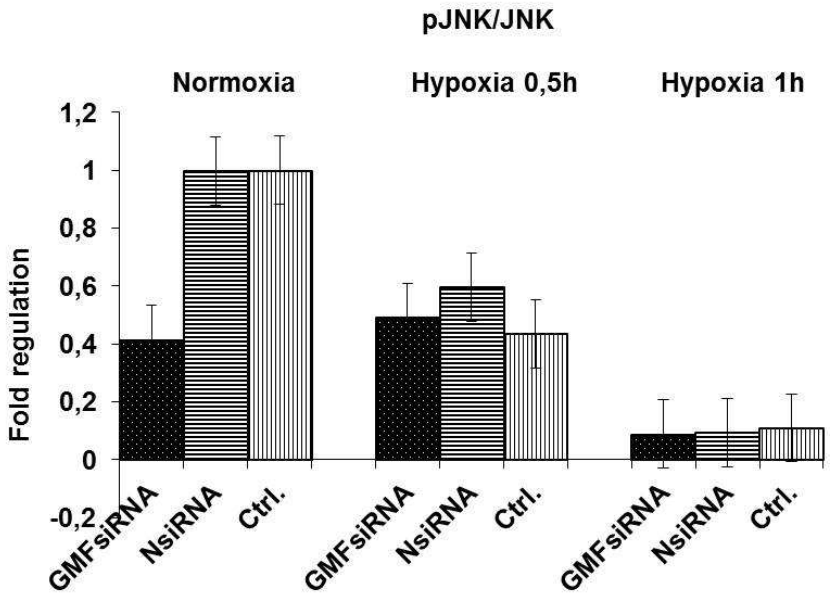


Figure 7:
a) phosphorylation of JNK detected by protein amount of pJNK is shown by means of western blot analysis above. In cells transfected with GMFsiRNA only under normoxic conditions phosphorylation is attenuated, after hypoxic treatment (0.5 h and 1 h) this effect is annulated and cells transfected with GMFsiRNA, NsiRNA and control cells (CTRl.) show almost same protein amount of pJNK. After exposure to hypoxia phosphorylation of JNK decreases in a time dependent manner. GAPDH serves as loading control.
b) Fold regulation of pJNK /JNK under normoxic conditions and hypoxia.

5. Activation of EGFR after downregulation of GMF under normoxic and short time hypoxic conditions

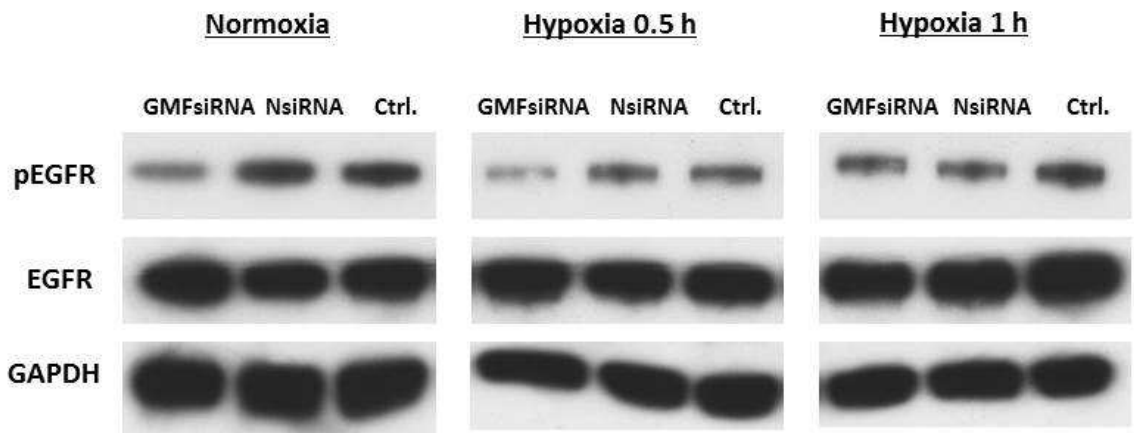
To date there is no evidence that there is any association between the epidermal growth factor receptor and expression of GMF. Because of the apparent relevance of EGFR for tumour genesis and disease progression interactions between GMF and deregulated pathways in tumour cells would be of highest interest for development of more efficient therapies.

To analyse a relevant interaction, LN18 glioblastoma cells were transfected with GMFsiRNA. Subsequently, they were subjected to hypoxia for 30 min or one hour or kept under normoxic conditions. Immediate protein isolation and Western Blot analysis was applied to determine protein levels of EGFR and phosphorylated e.g. activated EGFR (pEGFR).

Under normoxic conditions activated EGFR is detectable in the LN18 control cells.

Interestingly there was about 66% less phosphorylated protein of EGFR after downregulation of GMF protein in cells under normoxic conditions. Same results were observed following 30 min of hypoxia. After one hour under hypoxic conditions there was no relevant difference to phosphorylated EGFR in control cells (CTRl.) any more, the phosphorylation in GMFsiRNA, NsiRNA and CTRl. was slightly decreased.

a)



b)

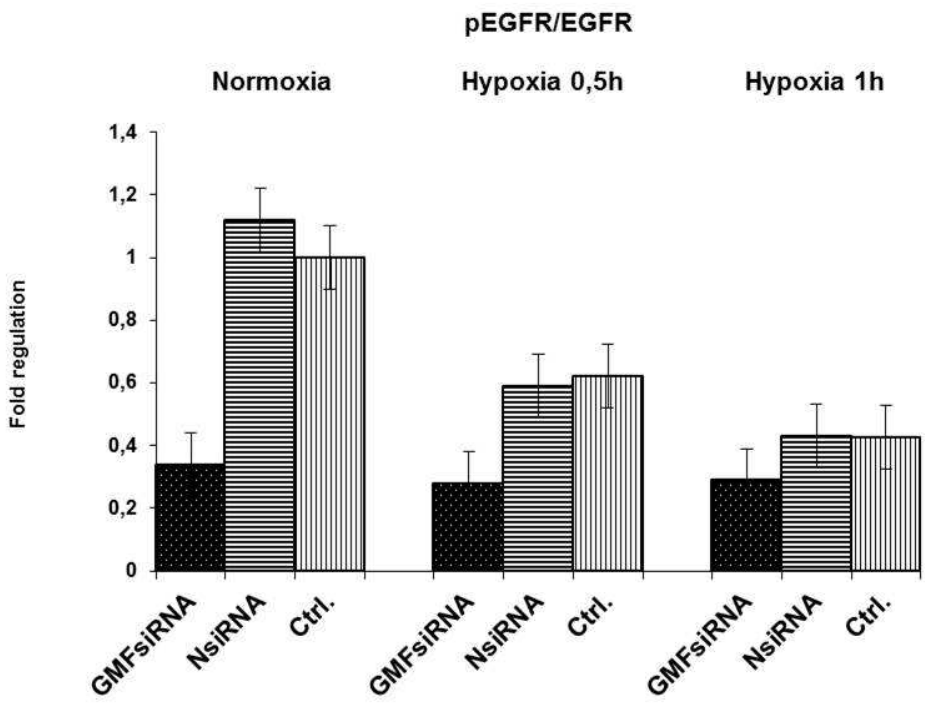
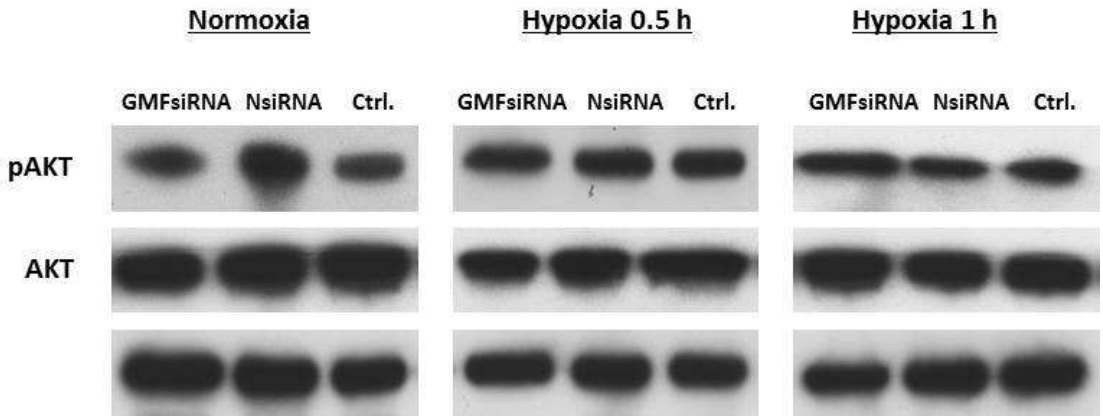


Figure 8:
a) Transfected cells (GMFsiRNA, NsiRNA) and control cells (CTRl.) were exposed to hypoxia for 30 min or one hour. Immediate protein isolation and evaluation by Western blot show protein levels of EGFR and phosphorylated EGFR (pEGFR). There is less phosphorylated protein of EGFR in cells under normoxic conditions and 30 min after downregulation of GMF protein. After one hour under hypoxic conditions phosphorylation of EGFR returns to the level as seen in the control cells. GAPDH serves as protein loading control.
b) Fold regulation of protein level of pEGFR /EGFR

6. Activation of AKT and ERK1/2 downstream of EGFR after downregulation of GMF under normoxic and short time hypoxic conditions

Two important pathways transferring signals from activated EGFR into the cells are the PI3K/AKT/mTor pathway and the MAPK/ERK1/2 pathway. Therefore transfected cells were immunoblotted with the indicated antibodies against the activated protein and whole protein amount of AKT and ERK1/2 after exposure to hypoxia or normoxia.

a)



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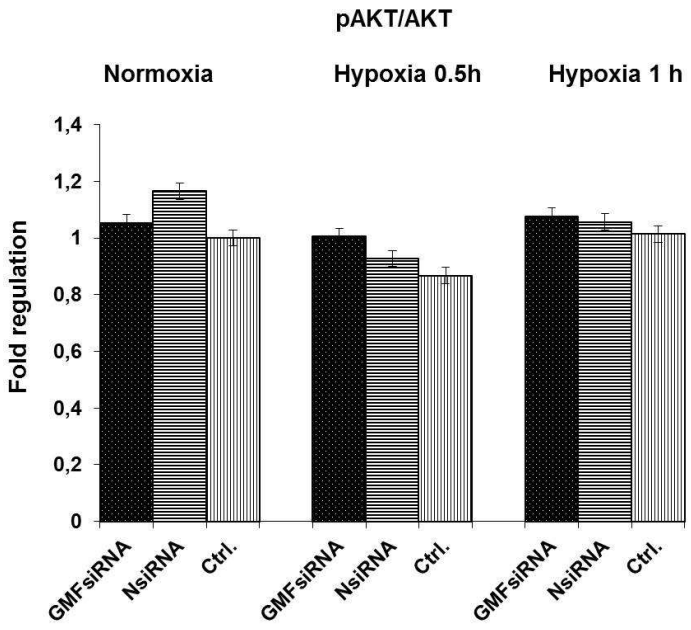
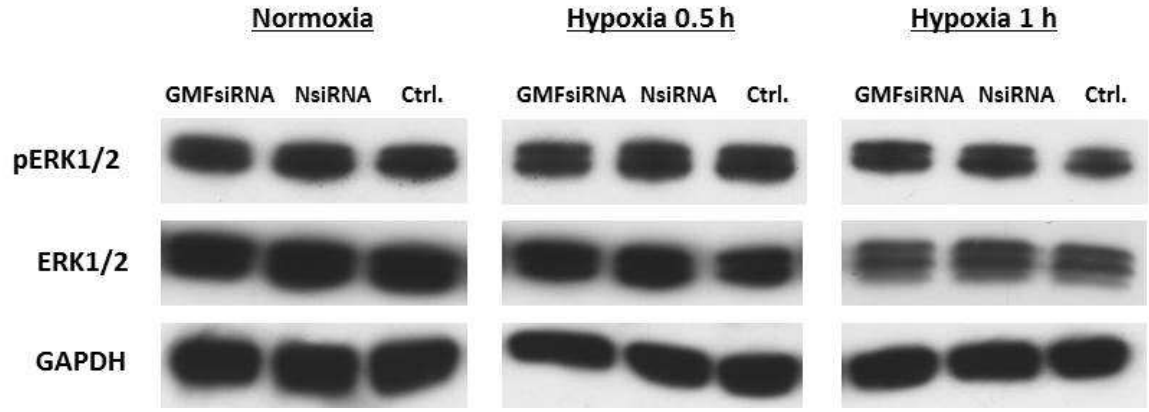


Figure 9:
a) Western blot analyses of protein level of AKT and pAKT did not show any change either after transfection with GMFsiRNA or after exposure to hypoxia. GAPDH serves as protein loading control.
b) Fold regulation of protein level of pAKT /AKT

There was a clear phosphorylation of AKT and ERK1/2 in control cells under normoxic conditions indicating for a constitutive activation of these pathways in LN18 cell line. This activation was not affected by transfection with GMFsiRNA nor hypoxic treatment.

a)



b)

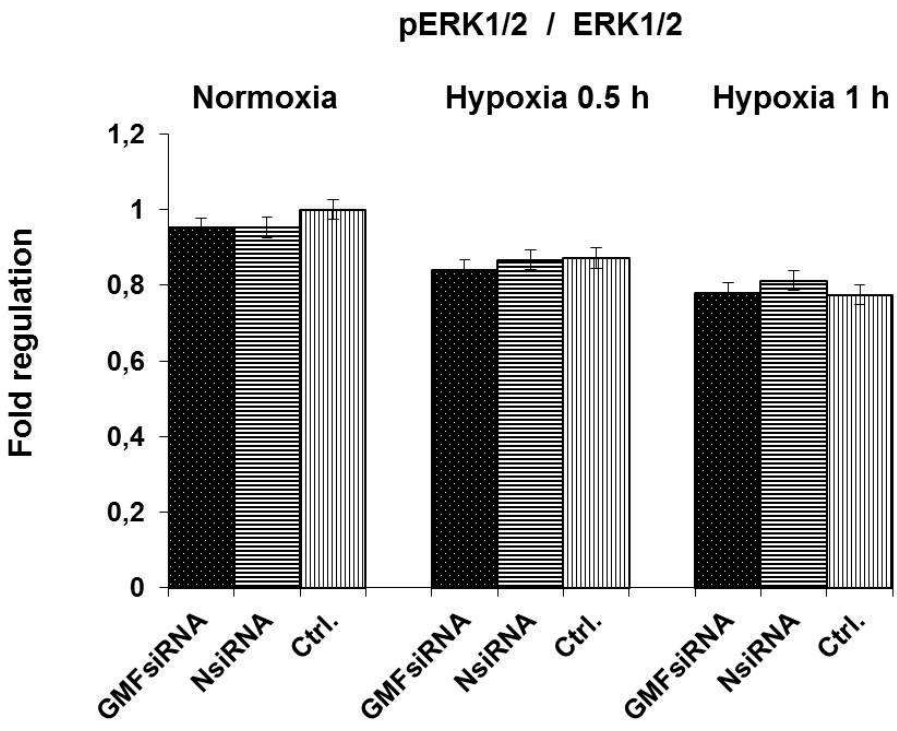


Figure 10:
a) Western blot analysis of protein level of ERK1/2 and pERK1/2 did not show relevant change both after transfection with GMFsiRNA and after exposure to hypoxia. GAPDH serves as protein loading control.
b) Fold regulation of pERK1/2 /ERK1/2 under normoxic conditions and hypoxia.

7. Regulation of relevant pathway and downstream targets after downregulation of GMF under hypoxia of six hours

The regulation of expression or activation of relevant pathway proteins were analysed by western blot analysis. Cells transfected with GMFsiRNA and control siRNA (NsiRNA) as well as not treated cells (CTRl.) were exposed to hypoxia for six hours. After six hours, all cells were tested for the expression of either whole protein (GMF) or phosphorylated e.g. activated protein (pEGFR, pAKT, pp38, pJNK, pERK).

There was no significant decrease of expression of GMF protein after six hours of hypoxia in control cells. The downregulation via specific siRNA held up.

Interestingly, the phosphorylation of EGFR, AKT and JNK was almost completely attenuated after exposure to hypoxia for six hours. In contrast there was still a considerable phosphorylation of p38 in cells transfected with NsiRNA and control cells after 6hrs under hypoxia, while attenuated phosphorylation of p38 following downregulation of GMF was still persistent. ERK1/2 was equally phosphorylated under normoxic and hypoxic conditions. As control for hypoxic conditions achieved in LN18 cells expression of HIF-1α was tested.

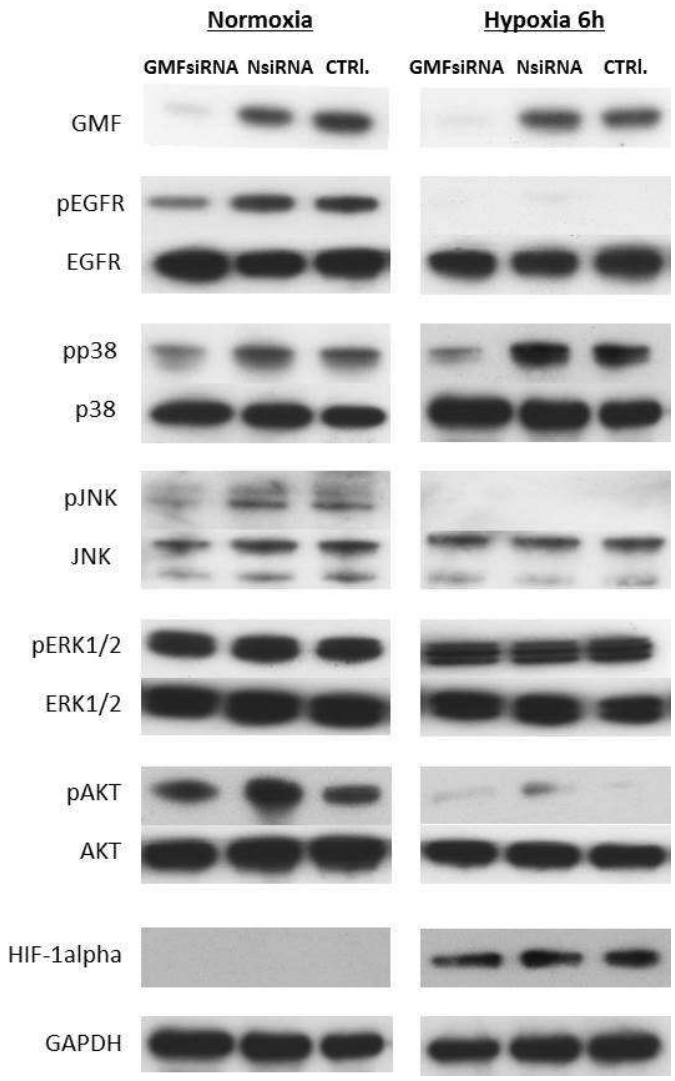


Figure 11: The regulation of expression or activation of relevant pathway proteins under hypoxic conditions of six hours are show by western blot analysis. After six hours all cells were tested for protein amount either whole protein (GMF) or phosphorylation status (EGFR, AKT, p38, JNK, ERK). There is no significant downregulation of expression of GMF protein after six hours of hypoxia. Interestingly the phosphorylation of EGFR, AKT, and JNK are almost off after longer exposure to hypoxia, only phosphorylation of ERK1/2 and p38 still persists. HIF-1α serves as indication of hypoxic conditions in cell, GAPDH serves as loading control.

An overview of regulated pathway transducers in the LN18 cells treated with siRNA against GMF under normoxia and hypoxia is given in Table 2 and Table 3.

Table 2: Overview of regulation of indicated activated protein depending on expression level of GMF under normoxic conditions. Legend: all arrows indicate changes in immunoreactivity compared to immunoreactivity with constitutive activation. Strong arrow = strongly changed level, thin arrow = slightly changed level, double arrow: not changed level.

Cell Line	GMF expression	pEGFR	pAkt	pERK 1/2	pp38	pMK2	pATF- 2	pJNK
LN18	+	const. activated	const. activated	const. activated	const. activated	const. activated	const. activated	const. activated
	-	↓	↔	↔	⇓	↓	⇓	↓

Table 3: Overview of regulation of indicated activated protein depending on expression level of GMF under hypoxic conditions. Legend: all arrows indicate changes in immunoreactivity compared to immunoreactivity found under normoxic conditions. Strong arrow = strongly changed level, thin arrow = slightly changed level, double arrow: not changed level.

Hypoxia time [h]	GMF expression	pEGFR	pAkt	pERK 1/2	pp38	pMK2	pATF-2	pJNK
0.5	+	↓	↔	↔	↑	↓	↑	↓
	-	↔	↔	↔	↔	↓	↔	↔
1	+	↓	↔	↔	⇑	⇓	⇑	⇓+
	-	↔	↔	↔	↔	⇓	↔	⇓
6	+	⇓	⇓	↔	↑	⇓	⇓	⇓
	-	⇓	⇓	↔	↔	⇓	⇓	⇓

D. Discussion

Glioblastoma is the most frequent and most malignant primary brain neoplasm in adults. Infiltrative growth and aberrant signal transductions render it highly resistant to current treatments including cytoreductive surgery, radiation therapy and chemotherapy (Hofer and Herrmann 2001). Furthermore the tumoural microenvironment seems to play a major role in tumour therapy resistance and invasion. In recent years, hypoxia has been implicated as a major environmental stimulus for these biological and clinical features (Rampling et al. 1994, Kaur et al. 2005). The inflammatory environment frequently affecting the tumour is another important finding that seems to be involved in the migration and invasion into surrounding brain tissue (Sen 2011).

The Glia Maturation Factor (GMF) is a brain dominant molecule involved in inflammatory brain processes. Data of the Zaheer group demonstrated GMF in the inflammatory cascades of Alzheimer's disease (Zaheer et al. 2011).

Unpublished work of our working group pointed to possible expression of GMF mRNA in glioma cells.

The aim of the present study focused on the expression of GMF at the protein level in different glioma cell lines and its potential regulation by stress signalling pathways. In addition, the role of hypoxia for the expression of GMF and for the activation of relevant pathways has been investigated.

Therefore the established glioblastoma cell line LN18 that showed high expression level of GMF protein under normoxic conditions was chosen. Because there was no assay with specific antibody against pGMF, the investigation of functional effects of GMF was performed by downregulation of GMF mRNA by specific siRNA technology. The role of hypoxia was investigated by GBM cells growth under hypoxic conditions of 0, 66% O_2. The functional regulation of GMF and its role in tumour growth has been analysed at the protein level by Western Blot analyses using specific GMF antibodies.

The major results obtained by this approach included the following interesting observations:

a.) GMF is expressed in GBM cell lines. The highest expression level could be found in the glioma cell line LN18. These cells were therefore chosen for functional analyses. In LN18 cells a constitutive activation of different signalling pathways exists including p38, MK2, ATF-2, JNK, ERK1/2, EGFR and AKT under normoxic conditions.

b.) The p38 MAPK pathway that plays a crucial role in inflammation is constitutively activated in LN18 cells. This activation was blocked by downregulation of GMF up to six hours of hypoxic treatment. In control cells the activation of p38 increased under hypoxic conditions.

c.) After downregulation of GMF by siRNA a diminished phosphorylation of the EGFR under normoxic conditions could be observed. Compared with untransfected and mock-transfected control cells that show a downregulation of EGFR by hypoxia, no alteration could be found in LN18 cell with GMF downregulation. Although GMF seems to be involved in EGFR activation, no effect could be detected in EGFR downstream substrates including ERK1/2 and AKT.

1. Knockdown of GMF, Normoxic conditions

GMF is a brain dominant protein which has been shown to interact with the p38 MAPK and neuronal inflammatory processes. GBM is inflammatory by nature (Yeung et al. 2012). To detect relevant interactions under normoxic conditions, GMF was downregulated by siRNA technology in LN18 cells. Relevant proteins (EGFR, AKT, ERK1/2, JNK, p38, MK2, ATF-2) were tested at the protein level and for activation by phosphorylation using specific antibodies; immunoreactivity was detected by Western Blot Analyses.

1.1. Attenuated activation of p38 and JNK after downregulation of GMF under normoxic conditions

One important family of kinase cascades that mediate cellular reactions of stress, cytokines or growth factors is the MAP kinase family. Three distinct subgroups exist: extracellular signal-regulated kinases1/2 (ERK1/2), c-jun N-terminal or stress activated protein kinases (JNK/SAPK)

and p38 MAP Kinase. While ERK1/2 is activated by mitogenic stimuli, JNK and p38 MAPK are typically activated in response to cellular stress such as UV light, heat, osmotic shock and inflammatory cytokines and correlated mainly with enhanced apoptosis (Xia et al. 1995). In the Glioblastoma cell line LN18, a constitutive activation of p38 and its substrates MK2 and ATF-2 was found under normoxic conditions in the present study. One very important pathway for cell response to inflammatory stress e.g. production of inflammatory cytokines is the p38 pathway. Taking in account that an inflammatory microenvironment is regulary detected in glioblastomas the activation of the p38 pathway could contribute the inflammatory microenvironment of glioblastomas. In my experiments the downregulation of GMF diminished the phosphorylation (i.e. activation) of p38 and its substrate ATF-2 by more than 50 % while only a minor effect on MK2 was observed (about 30% less). These results are in accordance with former work that already illuminated the effect of GMF on the p38 signalling pathway in other cell lines. (Zaheer et al. 2007). In the present study downregulation of GMF protein correlates with impaired activation of p38 pathway in LN18 glioblastoma cells. It can be hypothesized that the decreased activation of p38 attenuates production of pro- inflammatory cytokines, which have been shown to support migration and invasion in carcinogenesis (Sen 2011). In support to this hypothesis the group around Yeung could demonstrate, that p38 inhibitors diminish production of pro- inflammatory cytokine production resulting in decreased invasiveness in human U251 glioblastoma cells (Yeung et al. 2012).

Another MAPK, JNK, is activated in response to a variety of environmental stresses and cytokines. It takes an important part in cellular proliferation and survival. Recent studies showed that activated JNK in C6 cells implicated inhibition of proliferation and elevated apoptosis (Koyuturk et al. 2004). It is notable that JNK can exert completely opposite functions depending on the cell type and stimuli (Liu and Lin 2005). JNK is important for handling cellular stress such as induced by treatment with the chemotherapeutic drug temozolomide, which makes part of the standard treatment of glioblastoma. The group around Ohba et al. 2009 observed for glioma cells, that the cytotoxicity induced by temozolomide, a DNA-alkylating agent, is potentiated by a JNK inhibitor (Ohba et al. 2009). Here I found for the LN18 cell line activated JNK under normoxic conditions without external stimuli. As discovered for p38, JNK activation was as well

impaired following downregulation of GMF protein. Concerning the anti-apoptotic properties JNK seems to have in the glioma cells this could be the function of the constitutive activation of JNK observed in the cell line LN18 in the present study as well.

1.2. Attenuated activation of EGFR but not AKT or ERK1/2 after downregulation of GMF under normoxic conditions

EGFR mutations, amplification and overexpression are often observed in human glioblastomas. Under normoxic conditions EGFR signalling enhances proliferation and migration; furthermore it has anti-apoptotic properties (Lund-Johansen et al. 1990, Steinbach et al. 2002). In the LN18 cell line examined in the present study a constitutive activation of EGFR under normoxic conditions was observed. Interestingly in cells transfected with GMFsiRNA EGFR activation was strongly decreased. The attenuated activation of EGFR could be due to the transfection conditions. Therefore an inactivation of the anti-apoptotic properties that were described for EGFR signalling through dephosphorylation could be the reason for this phenomenon (Yarden and Sliwkowski 2001). However, attenuated activation could not be observed in control cells.

Another interpretation could be predicated on the functional role of GMF in inflammatory processes. GMF- knock-out mice showed decreased pro-inflammatory cytokine expression like TNF-α and IL-1 (Zaheer et al. 2007). Furthermore it has been shown by Singhirunnusorn that a cross interference between TNF-α and EGFR signalling pathway exists (Singhirunnusorn et al. 2007). TNF-α induced phosphorylation of EGFR through the p38 signalling pathway in HeLa cells. This activation was maintained for 30 min. Turnover of p38 activation led to dephosphorylation of EGFR. At the same time TNF-α suppressed the ability of EGFR to respond to its extracellular ligands (Singhirunnusorn et al. 2007).

The results of the present study that show attenuated activation of EGFR might indicate that downregulated GMF subsequently attenuated TNF-α expression via p38 pathway in LN18 cells; this decreased expression resulted in dephosphorylation of EGFR.

EGFR mediates its effect on the cells mainly through the RAS- RAF- ERK1/2 pathway and the PIP3-Akt pathway.

While the latter pathway plays a pivotal role in cell survival, regulation of proliferation, migration and expression of angiogenic factors, ERK1/2 mainly mediates glioma cell growth and differentiation. Both AKT and ERK1/2 pathways have been linked to tumour progression of gliomas and dispose anti-apoptotic properties (McLendon et al. 2007). PIP3 is negatively regulated by PTEN. Loss of PTEN activation results in attenuated inhibition of proliferative pathways with following aberrant proliferation through constitutive activated AKT.

It has been demonstrated, that LN18 cells do not harbour *PTEN* mutations (Ishii et al. 1999). Despite this, however, there was constitutive activation of AKT in LN18 cells in the present study. Interestingly the constitutive phosphorylation of AKT was not affected by downregulation of GMF and lower activation status of EGFR. Probably the activation of this pathway is independent of both the activation of EGFR found in the LN18 cell line and downregulation of GMF protein. This hypothesis is in line with former studies on LN18 cells which also found constitutive activation of AKT (Sudheerkumar et al. 2008). The same result was obtained regarding the MAP Kinase pathway of ERK1/2 in the present study, another important signalling pathway transmitting the activation of EGFR into the cells. MAPK42/44, also known as ERK1/2, was found to be activated in the LN18 cell line under normoxic conditions. Downregulation of GMF had no influence on the activation of ERK1/2.

2. Knockdown of GMF, hypoxic conditions

Jensen et al. could demonstrate a correlation to tumour progression and hypoxia. Hypoxia is thought to be the mechanism by which an aggressive tumour phenotype is developed through increased invasion, loss of apoptosis and therapy resistance (Jensen 2009). To clarify alterations or activations of relevant pathways in hypoxic environment, LN18 cells were subjected to hypoxia of about 0.66 % O_2 after transfection with GMFsiRNA. Relevant temporal changes of activation levels of interested proteins were tested after different hypoxia times (30 min, 1 h and 6 hrs.); as environment control cells were kept under normoxic conditions.

2.1. Blocked activation of p38 under hypoxic conditions only after transfection with GMFsiRNA, decreasing activation of JNK

Recently Wang et al. examined the activation of MAP kinase pathways p38, JNK and ERK1/2 under hypoxic conditions over 24h (Wang et al. 2009). They showed that in neuroblastoma cells under moderate hypoxic conditions (3%) that the phosphorylation of p38 decreased in a time dependent manner and after one hour activated p38 was not detectable any more. Activation of ERK1/2 was biphasic with a peak at 60 min, returning to baseline after 6 h and again increasing up to 12 hours, JNK had a considerable peak at 3 hours and then returned to baseline. Furthermore they observed hypoxia- induced upregulation of the vascular endothelial growth factor (VEGF) during the hypoxic treatment. Once activated, the receptor of VEGF is known to effect vascular permeability, migration, and proliferation of endothelial cells. The assumption of that study was that VEGF mediates its anti-apoptotic and protective properties under hypoxia via ERK1/2. P38 was rapidly dephosphorylated in the neuroblastoma cells. It was suggested that this deactivation could provide neuroprotection. Moreover the deactivation and downregulation of JNK after six hours of hypoxic treatment might protect the neuroblastoma cells from apoptosis. Besides JNK may indirectly regulate HIF-1-mediated transcription of VEGF (Wang et al. 2009).

In the present study GMF protein was still highly expressed after six hours of hypoxia and downregulation by GMFsiRNA persisted. For the p38 signalling pathway our findings were notable diverse referring to the work of Wang et al.. These results may outline the variable role hypoxia plays depending on both cell type and the degree of hypoxia (0, 66% in the present study). There was a distinct increased activation of p38 up to one hour under hypoxic conditions; same results could be shown for its nuclear substrate ATF-2. However, MK2 activation decreased under hypoxia after one hour. After six hours of hypoxia the activation of p38 in LN18 cells was slightly decreased but still detectable, activation of ATF-2 and MK2 was no longer observed. In cells transfected with GMFsiRNA activation of p38 remained blocked. GMF seems to play a pivotal role in the activation of p38 signalling pathway under hypoxia in LN18 glioblastoma cells. After one hour of hypoxia control cells with physiological level of GMF protein had an increase of activated p38 about 2-fold more than under normoxic conditions.

Transfection with GMFsiRNA disabled this additional activation and phosphorylated p38 remained on the same lower level as under normoxic condition. In inflammatory cell responses GMF plays an important role (Zaheer et al. 2007). Concerning the strong increase of activated p38 it can be hypothesized that under short time hypoxic condition the inflammatory cell response becomes more important for the glioblastoma cells; moreover after six hours there was still a notable activation of p38 suggesting the close relationship of both hypoxia and inflammation in the microenvironment of LN18 cells. Therefore GMF could be an important promoter. With downregulated GMF the LN18 cells did not show activated p38 as cells with physiological level of GMF.

However the MAP kinase JNK differs in activation compared to p38. The phosphorylation decreased clearly in a time dependent manner under hypoxic treatment. JNK is activated by various extracellular stimuli and then translocated to the nucleus, where it phosphorylates and activates transcriptional factors. Inhibition of JNK and subsequent inhibition of phosphorylation of c-Jun leaded to enhanced temozolomide- induced cytotoxicity in human glioma cells. In contrast in C6 glioma cells inhibiting JNK partly protected the cells from apoptosis induced by a lipid- lowering drug (Simvastatin) (Koyuturk et al. 2004, Ohba et al. 2009). These publications accentuate the important and opposing roles JNK is playing in regulation of cell death or cell survival. In the present work JNK is rapidly dephosphorylated and after six hours of hypoxic treatment no activation was observed. The assumption could be that this deactivation protects the LN18 cells from hypoxia induced apoptosis.

Obviously, various stressors can have different effects, depending on the specific cell type and its environment. The different effects of hypoxia on p38 and JNK contribute to a small number of stimuli that selectively activate p38 but not JNK (Wang and Proud 1997). It is well known that both p38 and JNK phosphorylate ATF-2 (Vlahopoulos et al. 2008). Considering the increasing activation both p38 and ATF-2 it can be assumed, that in the present work under short time hypoxic conditions the phosphorylation of ATF-2 is mainly a result of activation by p38 and not JNK. Accordingly after six hours phosphorylation of ATF-2 decreased what stands for an early response to hypoxic conditions. Activation of p38 holds up till six hours under hypoxic conditions. This suggests other important targets of p38 under special conditions such as

tumour hypoxia. While tumour cells die and the tumour develops necrotic areas the inflammatory environment might support increasing invasiveness and surviving of tumour cells under hypoxic conditions. This important mechanism might be promoted by activated p38, which was found as constitutive activation under normoxic conditions but also after six hours after hypoxic treatment. Concerning the activation of p38 in LN18 cells after hypoxic treatment it could be interesting to put more attention in subsequent research on appropriate molecules that can affect the p38 pathway.

2.2. Decreased activation of EGFR and AKT, no effect of phosphorylation of ERK1/2 after treatment with hypoxia

In the present study it could be observed that after transfection with GMFsiRNA phosphorylation of EGFR was decreased under normoxic conditions; in control cells a constitutive activation of EGFR was observed. After one hour under hypoxic conditions the activation of EGFR decreased; in addition the effect of attenuated activation of EGFR found under normoxic conditions in cells with downregulated GMF was abolished. After six hours of hypoxia there was no phosphorylated EGFR detectable anymore whereas the whole EGFR protein level was unchanged. Activation status of AKT and ERK1/2 were completely independent of downregulation of GMF both after hypoxic treatment and under normoxia.

After one hour under hypoxic conditions phosphorylated proteins of AKT and ERK1/2 were almost the same compared with normoxic conditions. Interestingly after 6 hours of hypoxia activation of AKT decreased dramatically without changes of whole AKT protein level, while phosphorylation of ERK1/2 was consistently high. The decreased activation of EGFR and AKT observed in the present study under hypoxic conditions is in line to the finding of Steinbach et al. (Steinbach et al. 2002). They demonstrated in glioma cell lines that inhibition of EGFR protected cells from cell death induced by acute hypoxia. Hypoxia induced cell death was determined by lactate dehydrogenase release, depletion of glucose and ATP. The combined inhibition of PI3K and ERK1/2 mimicked the protective effect of inhibited EGFR. After inhibition of PI3K or ERK1/2 alone only the inhibition of PI3K reduced glucose consumption to a similar degree as inhibition of EGFR (Steinbach et al. 2002).

The constitutive activation of EGFR and AKT found in the present study for LN18 cells under normoxic conditions could bring the tumour malignant benefit such as aberrant progression and survival; however under hypoxic conditions EGFR and AKT get “deactivated”; this could protect LN18 cells from hypoxia induced cell death as demonstrated by Steinbach et al (Steinbach et al. 2002). Strikingly at the same time activation of ERK1/2 remained equally high after subjection under hypoxia like it was under normoxia. This could point to an important role of ERK1/2 under hypoxic conditions. In the neuroblastoma cell line SH-SY5Y it could be shown that ERK1/2 promotes cell proliferation under hypoxia (Fang et al. 2010). Furthermore, different work demonstrated the critical role of ERK1/2 on proliferation under hypoxic conditions in different cell lines such as hypoxia induced phosphorylation of ERK1/2 and increased proliferation of U87MG glioma cells (Zhang et al. 2012). In addition repeated hypoxic exposures selected glioblastoma cells which expressed higher levels of phosphorylated ERK1/2 and showed more invasive potential (Kim et al. 2009). These important characteristics could be the reason for the on-going high activity of ERK1/2 found in the LN18 glioblastoma cell line. While attenuated phosphorylation of EGFR and AKT might protect the cell for hypoxia induced cell death, active ERK1/2 might promote strongly the invasiveness and proliferation.

3. Conclusion

Glioblastoma is one of the highest lethal and malignant adult neoplasms. Until today all efforts to find effective therapeutic strategies remain moderate. Different reasons for the clinical behaviour of GBM are discussed. Total surgical resection of this highly invasive tumour remains often incomplete. Moreover resistance of this tumour type to chemotherapy and radiation therapy occurs regularly. GBM are considerably heterogeneous, and the intratumoural diversity probable supports therapeutic resistance (Stupp et al. 2009). Beside this, the highly resistance rate of this tumour could be caused by the tumour microenvironment concluding inflammation and hypoxia and consequently the existence of highly resistant cells and tumour stem cells (Taylor et al. 2012). The present study investigated the correlation between expression of GMF (glia maturation factor) and the MAP Kinase pathways under normoxic and hypoxic conditions.

Hypothetic models are shown in Figure 12.

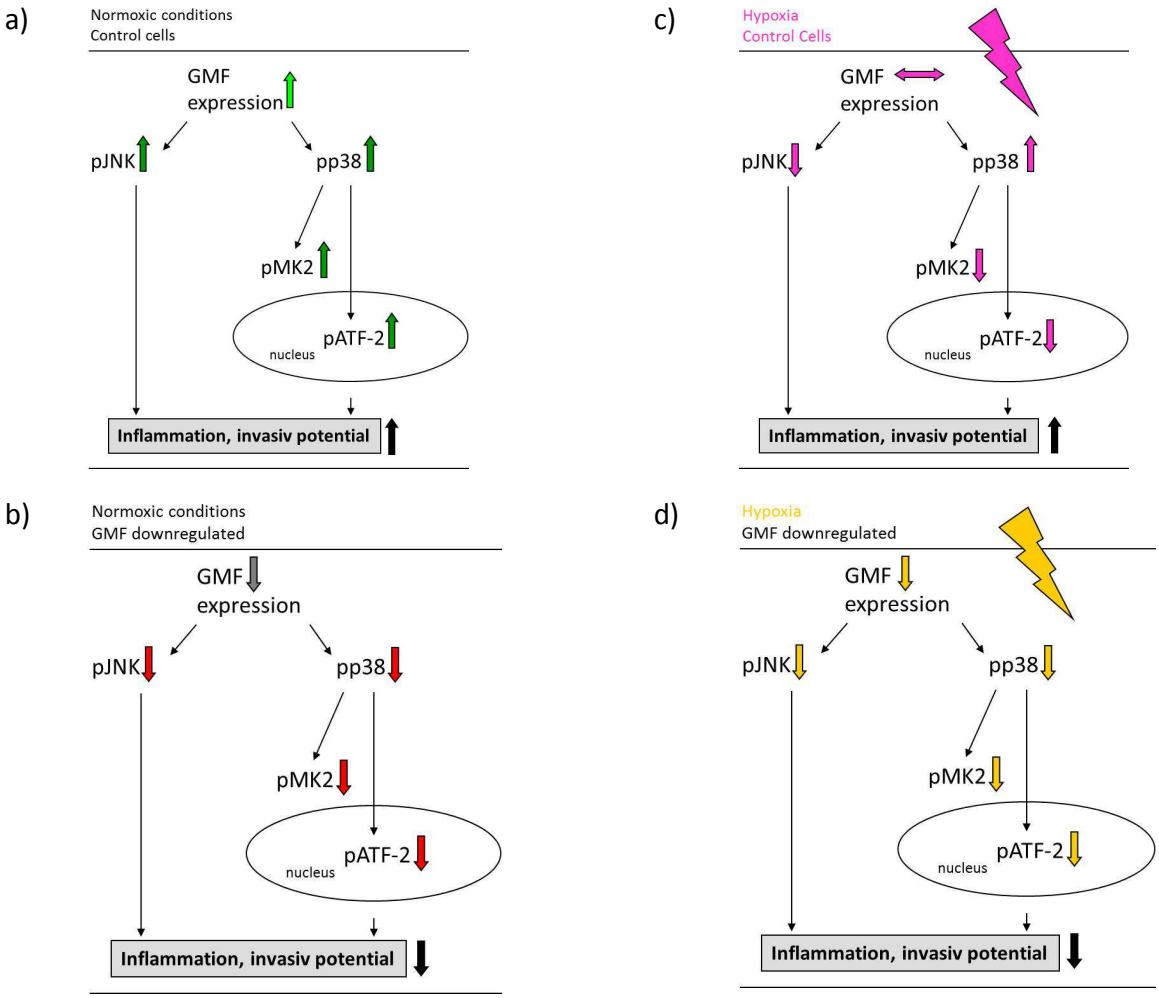


Figure 12:
a) Potential correlation in LN18 cells under normoxia
dark green arrows: constitutive activated protein
light green arrow: high expression level;
black thick arrow: potential impact on cell characteristics.
b) Potential correlation in LN18 cells under normoxia after downregulation of GMF
grey arrow: low expression level
red arrows: changes in activated protein level after downregulation of GMF
black thick arrow: potential impact on cell characteristics
c) Potential correlation in LN18 cells after 1 h of hypoxia
pink arrows: changes in activated protein level in control cells;
black thick arrow: potential impact on cell characteristics
d): Potential correlation in LN18 cells after 6 hrs. of hypoxia
orange arrows: changes of activated protein level after downregulation of GMF protein
black thick arrow: potential impact on cell characteristics.

E. Summary

Glioblastoma (GBM, WHO grade IV) is the most frequent primary brain tumour and the prognosis is still poor. Characteristics of this highly malignant tumour are infiltrative growth and regular recurrence. The clinical behaviour of the tumour reflects the biological properties of the tumour cells. The identification of the molecular key players is a prerequisite for the development of novel targeted therapeutic strategies.

In the present study the expression of Glia Maturation Factor (GMF) in human GBM could be demonstrated.

GMF is a brain dominant protein that plays an important role in inflammatory processes. The functional role of GMF in human GBM cells was investigated by downregulation of GMF by siRNA. Using this approach p38 reaction to hypoxia was completely abolished for up to six hours under hypoxic conditions. Consequently, the downstream target of p38, the activation transcription factor-2 (ATF-2) also showed no response to hypoxia. In normal LN18 cells and in mock-transfected cells p38 and ATF-2 were immediately activated by hypoxia. Also, a diminished activation of the EGFR could be shown in GMF downregulated cells when compared with untransfected cells, but only under normoxic conditions.

The data presented here show a role for GMF in hypoxia response in human glioblastoma cells and could therefore open new insights into the molecular regulation of brain tumour cells to micro environmental stress.

Zusammenfassung

Das Glioblastom (GBM, WHO Grad IV) ist der häufigste hirneigene Tumor beim Erwachsenen und hat immer noch eine infauste Prognose. Seine hochmalignen Eigenschaften gehen vor allem auf sein infiltratives Wachstum und die regelmäßig vorkommenden Rezidiven zurück. Das klinische Erscheinungsbild eines Tumors spiegelt das molekulare Verhalten der Tumorzellen wider. Eine wichtige Voraussetzung für neue Therapiestrategien ist daher die Ermittlung von Molekülen, die eine Schlüsselrolle in der Entwicklung der hochmalignen Eigenschaften einnehmen.

In der vorliegenden Arbeit konnte gezeigt werden, dass Glioblastomzellen den Glia Maturation Factor (GMF) exprimieren. Die funktionelle Rolle des GMF in den Glioblastomzellen wurde durch Herabregulation von GMF durch spezifische GMFsiRNA untersucht. Durch diesen Ansatz wurde die Reaktion von p38 auf Hypoxie bis zu sechs Stunden komplett aufgehoben. Konsequenterweise zeigte auch das Substrat von p38, der Activating Transcription Factor-2 (ATF-2) keine Reaktion auf Hypoxie mehr. In normalen LN18 Zellen und in mock-transfizierten Zellen, wurden durch Hypoxie sowohl p38 als auch ATF-2 umgehend aktiviert. In den Zellen, in denen GMF herab reguliert wurde, konnte auch -verglichen mit nicht transfizierten Zellen- eine verminderte Aktivierung des Epidermal Growth Factor Rezeptors (EGFR) nachgewiesen werden, allerdings nur unter Normoxie.

Die vorliegenden Daten weisen auf eine Rolle von GMF unter sauerstoffarmen Bedingungen in menschlichen Glioblastoma Zellen hin und könnten damit neue Erkenntnisse über die molekulare Regulation von Tumorzellen im Gehirn unter schwierigen Umgebungsbedingungen liefern.

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Declaration of honour – Erklärung

Ich erkläre an Eides statt, dass ich die der Fakultät für Medizin der Technischen Universität München zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Expression of Glia Maturation Factor in human glioblastoma cells and its impact on cellular stress response after hypoxia

in der Fachabteilung Neuropathologie des Instituts für Allgemeine Pathologie und Pathologische Anatomie des Klinikums Rechts der Isar unter der Anleitung und Betreuung durch Univ.-Prof. Dr. med. J. Schlegel ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 5 angegebenen Hilfsmittel benutzt habe. Ich habe die Dissertation in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt. Ich habe den angestrebten Doktorgrad noch nicht erworben und bin nicht in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert. Die Promotionsordnung der Technischen Universität München ist mir bekannt.