Department of Radiation Biology

FACULTY OF HEALTH AND MEDICAL SCIENCES
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PhD thesis

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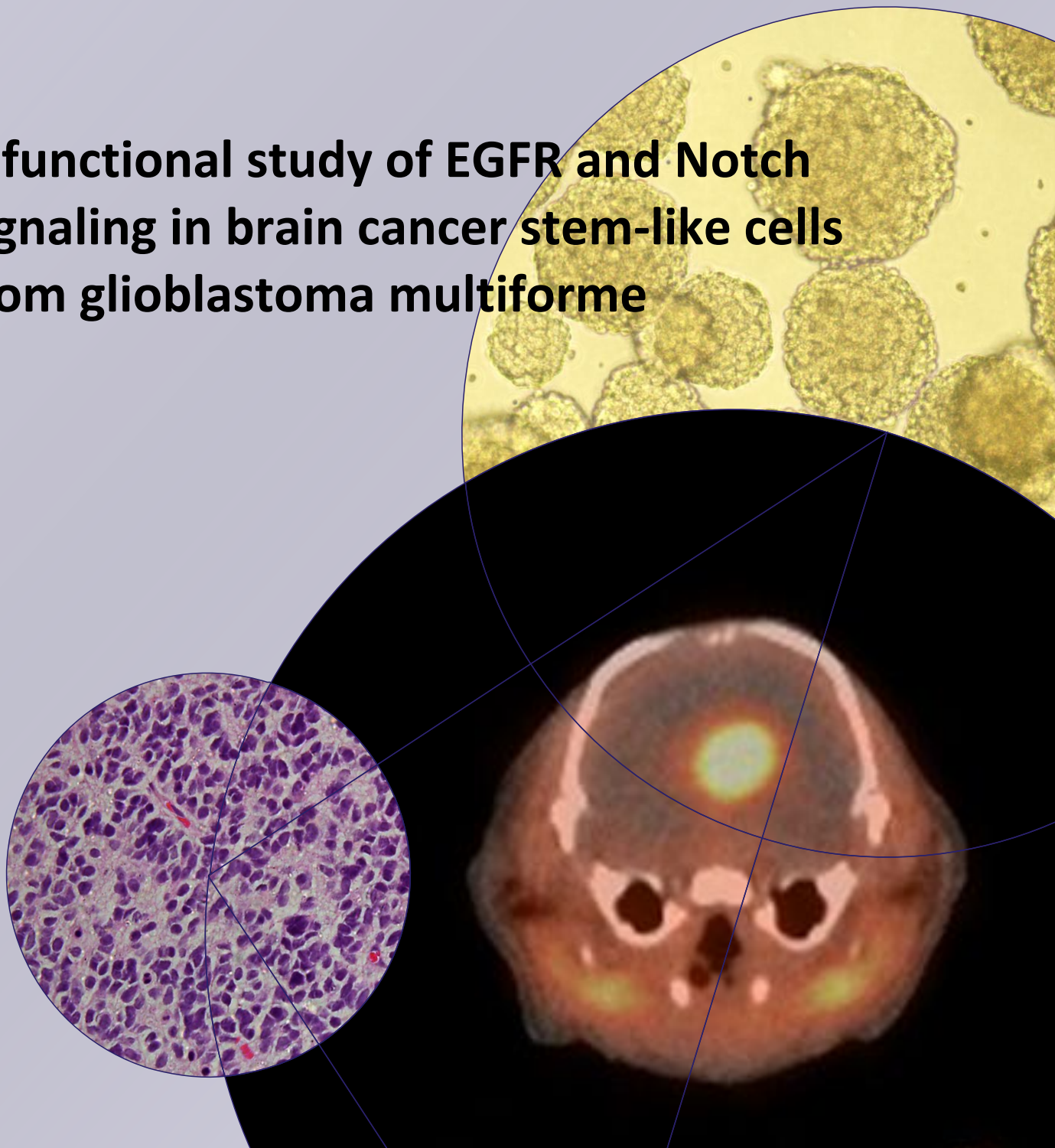
A functional study of EGFR and Notch signaling in brain cancer stem-like cells from glioblastoma multiforme



“In questions of science, the authority of a thousand
is not worth the humble reasoning of a single individual”

Galileo Galilei

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Preface

This thesis, entitled "A functional study of EGFR and Notch signaling in brain cancer stem-like cells from glioblastoma multiforme" is submitted to the Faculty of Health and Medical Sciences at the University of Copenhagen, Denmark, in order to fulfill the requirements for obtaining the PhD degree in health and medical science.

The work was carried out in the period from January 2010 to March 2013 under the supervision of Principal Supervisor, Head of Department, MD, DMSc Hans Skovgaard Poulsen, Project Supervisor MSc, PhD Marie- Thérése Stockhausen, at the Department of Radiation Biology, Copenhagen University Hospital, Denmark, and Co-Supervisor MD, PhD Ulrik Lassen, Department of Oncology, Copenhagen University Hospital, Denmark.

The majority of the experiments were conducted at the Department of Radiation Biology, The Finsen Center, Copenhagen University Hospital. The animal experiments were carried out at the Department of Experimental Medicine, The Faculty of Health and Medical Science, University of Copenhagen. The flow cytometric analyses were conducted at the Bartholin Institute, Copenhagen University Hospital. The immunohistochemical staining were performed at the Erler Group, the Biotech Research & Innovation Centre, University of Copenhagen, with technical assistance from the Department of Neuropathology, Copenhagen University Hospital. Finally, the global gene expression experiments and analyses were conducted at the Center for Genomic Medicine, Copenhagen University Hospital.

Financial support was received from The Faculty of Health Sciences, University of Copenhagen (211-0610/09-3012); The Danish Cancer Society; Dansk Kraeftforsknings Fond; Kong Christian den Tiendes Fond; Civilingenioer Frode V. Nyegaard og Hustrus Fond; Harboefonden; Danish Graduate School in Clinical Oncology; and Copenhagen University Hospital.

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Family and friends, I love you, simply for being there, supporting me and putting up with my narrow and preoccupied mind in particular the last couple of months. Just because you´re not mentioned doesn´t mean you are forgotten ☺

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To you all: THANK YOU

Karina Kristoffersen
Copenhagen, March 2013

List of papers

This thesis consists of four original manuscripts included in the results section and one review and one book chapter not included in the book edition of the thesis. The manuscripts are referred to in the text by their Roman numerals:

I Marie-Thérése Stockhausen, Helle Broholm, Mette Villingshøj, Maria Kirchhoff, Tommy Gerdes, Karina Kristoffersen, Michael Kosteljanetz, Mogens Spang-Thomsen and Hans Skovgaard Poulsen: ”Maintenance of EGFR and EGFRvIII expression in an *in vivo* and *in vitro* model of human glioblastoma multiforme”. *Experimental Cell Research*, 317(11): 1513-26, 2011

II Marie-Thérése Stockhausen, Karina Kristoffersen, Marie-Louise Stobbe Olsen and Hans Skovgaard Poulsen: “Differentiation of human glioblastoma multiforme stem-like cells leads to down regulation of EGFR and EGFRvIII expression and decreased tumorigenic and stem-like cell potential”. *Submitted.*

III Karina Kristoffersen, Mette Villingshøj, Marie-Thérése Stockhausen, Hans Skovgaard Poulsen: ”Level of Notch activation determines the effect on growth and stem cell-like features in glioblastoma multiforme neurosphere cultures”. *Cancer Biology & Therapy*, 14(7): 625-37, 2013

IV Karina Kristoffersen, Mette Kjølhede Nedergaard, Mette Villingshøj, Rehannah Borup, Andreas Kjær, Hans Skovgaard Poulsen and Marie-Thérése Stockhausen: ” Inhibition of Notch signaling alters the phenotype of orthotopic tumors formed from glioblastoma multiforme neurosphere cells but does not hamper intracranial tumor growth”. *Submitted.*

Marie-Thérése Stockhausen, Karina Kristoffersen, and Hans Skovgaard Poulsen: ”The functional role of Notch signaling in human gliomas”. *Neuro-Oncology*, 12(2): 199-211, 2010. (Not included in this edition)

Marie-Thérése Stockhausen, Karina Kristoffersen, and Hans Skovgaard Poulsen: ”Notch signaling in brain tumors”. Book chapter in: Reichrath J, Reichrath S red., “Notch signaling in embryology and cancer”. *Landes Bioscience,* s. 289-304, 2012. (Not included in this edition)

Dansk resumé

Glioblastoma multiforme (GBM) er den mest almindelige og aggressive hjernetumor hos voksne, med en median overlevelse for nydiagnosticerede GBM patienter på under 1,5 år. Trods en intensiv indsats i behandlingen, vil langt de fleste patienter opleve tilbagefald, og en stor del af forskningen i dag er derfor rettet mod nye molekylære og cellulære targets, der kan forbedre prognosen for GBM patienter. Et sådant target er de hjernecancer stamcelle-lignende celler (hCSC), som menes at være ansvarlige for tumor-initiering, -progression, behandlingsresistens og i sidste ende tilbagefald. hCSC identificeres på baggrund af deres lighed med normale neurale stamceller (NSC) og deres tumorigene potentiale. Som det er tilfældet for NSC, menes den epidermale vækstfaktor-receptor (EGFR) og Notch receptor signalering at være vigtig for opretholdelse af hCSC. På den baggrund udgør disse signaleringsveje et lovende target i en fremtidig anti-hCSC GBM behandling.

Det overordnede formål med dette PhD-projekt har været, at undersøge den funktionelle rolle af EGFR og Notch aktivitet i hCSCs stamcelle-lignende egenskaber og tumorigene potentiale med henblik på at uddybe vores viden omkring disse signaleringsveje i hCSC populationen i GBM.

Ved at etablere og dyrke humane GBM xenograft celler under NSC forhold opnåede vi neurosfære kulturer, der indeholdt celler med stamcelle-lignende og tumorigene egenskaber. Endvidere karakteriserede vi de forskellige kulturer baseret på deres ekspressionsniveau af EGFR og Notch receptoren samt ekspression af den muterede receptor EGFRvIII, en ekspression, der blev opretholdt fra patientmateriale til xenograft tumorer og cellekulturer. I en kultur, der overudtrykte EGFR samt udtrykte EGFRvIII, fandt vi, at EGFR inhibering førte til differentiering, mens forceret differentiering førte til nedregulering af EGFR og EGFRvIII. Derudover viste vi, at EGFR/EGFRvIII nedregulering, enten som følge af forceret differentiering eller EGFR inhibering resulterede i nedsat *in vitro* tumorigent og stamcelle-lignende potentiale. I kulturer, der udtrykte højt niveau af Notch-1 receptoren, fandt vi, at Notch inhibering nedsatte det *in vitro* tumorigene potentiale, mens det af de stamcelle-lignende egenskaber, kun var den primære sfære formation, der blev hæmmet. Kulturer med lav Notch ekspression blev ikke påvirket af Notch inhiberingen. Omvendt fandt vi, at kunstig aktivering af Notch signaleringen resulterede i øget *in vitro* tumorigent potentiale samt indikationer på et øget stamcelle-lignende potentiale i alle kulturer. Sammenlagt tyder disse *in vitro* resultater på, at aktiv EGFR og Notch signalering er vigtig for at opretholde hCSC populationens stamcelle-lignende og tumorigene potentiale. Da vi testede effekten af Notch inhibering på intrakraniel tumorvækst, observerede vi ikke øget overlevelse for mus injiceret med Notch-inhiberede celler, uanset cellernes oprindelige Notch aktivitet. Vi fandt imidlertid, at tumorer etableret fra kulturer med højt Notch udtryk og behandlet med en Notch inhibitor havde øget angiogent potentiale og en tendens til øget differentiering. Slutteligt fandt vi, at de neurosfære kulturer, der har været brugt i dette projekt, kunne yderligere karakteriseres på baggrund af deres globale gen-ekspressionsprofil, og at denne profil, i nogen grad, kunne korreleres med respons på Notch inhiberende behandling.

På baggrund af de, i dette projekt, opnåede resultater, mener vi, at det er muligt at targetere hCSC populationen ved hjælp af EGFR og/eller Notch inhibering og fremtidige studier vil vise om anti-hCSC behandling kombineret med den nuværende behandling kan forbedre prognosen for GBM patienter der udtrykker en specifik gen-ekspressionsprofil.

English summary

Glioblastoma Multiforme (GBM) is the most common and aggressive brain tumor in adults with a median survival for newly diagnosed GBM patients at less than 1.5 year. Despite intense treatment efforts the vast majority of patients will experience relapse and much research today is therefore searching for new molecular and cellular targets that can improve the prognosis for GBM patients. One such target is the brain cancer stem-like cells (bCSC) that are believed to be responsible for tumor initiation, progression, treatment resistance and ultimately relapse. bCSC are identified based on their resemblance to normal neural stem cells (NSC) and their tumorigenic potential. Like for NSC, the epidermal growth factor receptor (EGFR) and Notch receptor signaling pathways are believed to be important for the maintenance of bCSC. These pathways as such present promising targets in a future anti-bCSC GBM treatment.

The overall aim of the present PhD project has been to study the functional role of EGFR and Notch activity in bCSCs stem cell-like features and tumorigenic potential with the purpose of deepen our knowledge about the significance of these pathways in the bCSC population in GBM.

By establishing and culturing human derived GBM xenograft cells under NSC conditions we obtained neurosphere cultures that contained cells with stem cell-like and tumorigenic properties. We moreover characterized the different cultures based on their expression level of the EGFR and Notch receptor as well as the expression of the mutant receptor EGFRvIII, an expression that was maintained from patient material to the xenograft tumors and cell cultures. In a culture expressing EGFR and EGFRvIII we found that EGFR inhibition induced differentiation, while forced differentiation led to down-regulation of EGFR and EGFRvIII. In addition, we showed that EGFR/EGFRvIII down regulation either as a result of induced differentiation or EGFR inhibition led to decreased *in vitro* tumorigenic and stem cell-like potential. In cultures expressing high levels of the Notch-1 receptor we found that Notch inhibition decreased the *in vitro* tumorigenic potential while, of the stem cell features, only the primary sphere forming potential was inhibited. Cultures with low Notch expression were not affected by Notch inhibition. In opposite, we found that artificial Notch activation resulted in increased *in vitro* tumorigenic potential along with indications of increased stem cell-like potential in all cultures. Taken together, these *in vitro* results suggest that EGFR and Notch activity are important for maintaining the stem cell-like and tumorigenic potential of the bCSC population. When we tested the effect of Notch inhibition on intracranial tumor growth, we did not observe increased survival for mice injected with Notch inhibited cells regardless of the cells initial Notch activity. However, we found that tumors grown from high Notch expressing cultures treated with a Notch inhibitor displayed augmented angiogenic potential and a tendency to increased differentiation. Finally, we found that the neurosphere cultures used in this project could be further characterized based on their global gene expression profile and that this profile, to some degree could be correlated with response to Notch inhibitory treatment.

Based on the results obtained throughout this thesis project, we suggest that targeting a bCSC population by EGFR and/or Notch inhibition is feasible and future studies might prove if anti-bCSC therapy in combination with conventional therapy can improve the prognosis for GBM patients displaying a specific gene expression profile.

Abbreviations

ABC	ATP-binding cassette
AKT	"AK" was a temporary classification name for a mouse strain developing spontaneous thymic lymphomas. "T" stands for transforming, also known as protein kinase B (PKB)
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ASLV	avian sarcoma-leukosis virus (-A)
ATCC	american type culture collection
ATP	adenosine triphosphat
BBB	blood brain barrier
bCSC	brain cancer stem-like cells
bFGF	basic fibroblastic growth factor
bHLH	basic helix-loop-helix
CDKN2A	cyclin-dependent kinase inhibitor 2A
CoA	co-activator complex
CoR	co-repressor complexes
crh	chromosome
CSL	CBF, Suppressor of Hairless, LAG-1; also referred to as RBP-Jκ
Dlk-1	delta-like ligand-1
Dll	delta-like
D. Melanogaster	*Drosophila Melanogaster*
DMEM	dulbecco´s modified eagle medium
EC	endothelial cell
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ErbB/HER	avian homolog erythroblastic leukemia viral (v-erb-b) oncogene, human homolog named HER
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FDG	2-deoxy-2-^{18}F-flouro-D-glucose
FET	*O*-(2-[F]flouroethyl)-L-tyrosine
FLT	^{18}F-3'-fluoro-3'-deoxy-L-thymidine
Gab1	Grb2-associated protein 1
Grb2	Growth factor receptor-bound protein 2
GBM	glioblastoma multiforme
GDP	guanine dophosphate
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GSI	γ-secretase inhibitor
GTP	guanine triphosphate
HAT	histone acethyltransferase
Hash	human achaete-scute homologue

HB-EGF	heparin binding EGF-like growth factor
HDAC	histone deacetylase
Hes	hairy/enhancer of split
Hey	hairy/enhancer of split related with YRPW motif protein
i.v.	*intravenously*
ICN	intracellular Notch domain
ICN-1	intracellular Notch-1
IDH	isocitrate dehydrogenase
IHC	immunohistochemistry
Jag	jagged
LIF	leukemia inhibitory growth factor
LOH	loss of heterozygosity
loxP	locus of X-over P1
mAbs	monoclonal antobody (chimeric)
MAML	mastermind-like
MAPK	mitogen activated protein kinase, also named ERK
Mash	mammalian achaete-scute homologue
MEK	MAPK/ERK kinase
MGMT	*O*-6-methylguanine-DNA methyltransferase
miR	micro-RNA
MRI	magnetic resonance imaging
mTOR	Mammalian target of rapamycin
NB	neurobasal media with defined additives
NES	nestin
NF1:	neurofibromin 1
NMRI-nu	spontaneous mutant t-cell deficient mice
NSC	neurol stem cells
OX	orthotopic xenograft
PBT	primary brain tumor
PDGF:	platelet derived growth factor
PDK1	3-phosphoinositide dependent protein kinase-1
PET	positron emission tomography
PI3-K	phosphatidylinositol 3-kinase
PIP-2	phosphatidylinositol (4,5)-bisphosphate
PIP-3	phosphatidylinositol (3,4,5)-trisphosphate
PKB	protein kinase B
PTEN	phosphatase and tensin homolog
RA	*all*-trans-retinoic acid
RAF	serine/threonine kinase
RAS	GTPase
RCAS	replication competent ASLV long terminal repeat with splice acceptor
RNAi	RNA interference

RT	radiation therapy
RTK	receptor tyrosine kinase
SCID	severe combined immunodeficiency (spontaneous mutant T & B cell deficient mice)
SHH	sonic hedgehog
siRNA	small interfering RNA
SVZ	subventricular zone
SOS	son of sevenless
SX	subcutanous xenograft
T-ALL	acute T cell lymphoblastic leukemia
TCGA	the cancer genome atlas
TGF-α	transforming growth factor α
TKI	tyrosine kinase inhibito
TMZ	Temodal®, temozolamide
TVA	member of the low-density-lipoprotein receptor family, encoded by the *tv-a* gene and acts as the receptor for ASLV-A in avian cells
VEGF	vascular endothelial growth factor inhibitor
VZ	ventricular zone
WB	western blot
WHO	world health organization

Table of contents

1. Introduction

Brain tumors are among the most lethal malignancies in adults. They can be of primary-, intracranial origin, or secondary-, metastatic origin. Primary brain tumors (PBT) are classified according to the World Health Organization (WHO) as either low-grade (non-anaplastic, WHO Grades I-II) or high-grade (anaplastic, WHO Grades III-IV). PBT are mainly of neuroepithelial (neuroectoderm) origin and are traditionally distinguished based on their histological appearance of which gliomas is the most common PBT (50-70%) with a yearly incidence of approximately 5.5/100,000 in western countries[4,5]. Gliomas include oligodendrogliomas, mixed oligoastrocytomas, ependymomas and astrocytomas, whereof the most malignant form, the astrocytic glioblastoma multiforme (GBM, WHO Grade IV) accounts for approximately 70% of all high-grade gliomas[4]. In Denmark 900-1000 people are affected with PBT every year, 50% are gliomas and of them, approximately 260 are GBM[6,7]. In western countries the yearly incidence of GBM is 3.5/100,000[4] and the incidence has been increasing since the 1960s, probably due to refined diagnostic tools[8]. Treatment of GBM today consists of debulking surgery followed by chemo- and radiotherapy[9]. But despite this multimodal treatment the vast majority of patients experience relapse[10], thus GBM is still considered incurable and new treatments are in urgent need.

Today, much anti-GBM research is focusing on finding new targets that play a role in tumor formation and relapse. One such target is the so called brain cancer stem-like cells (bCSC). They are a population of cancer cells that shows great resemblance to normal neural stem cells (NSC)[11,12] and display resistance towards standard chemo- and radiation therapy[13,14]. They furthermore harbor angiogenic potential and tumorigenic ability[15-17]. One way to target the bCSC population could be through pathways known to be important for the normal NSC. Examples of these are the epidermal growth factor receptor (EGFR) and Notch signaling pathways which have been shown to play a role in both NSC and bCSC. EGFR is expressed in NSC, where it is involved in the activation of several downstream intracellular signaling pathways, which in turn regulate multiple cellular processes, such as proliferation, migration and survival[18]. Notch signaling is mediated through the Notch receptors, that likewise are expressed in NSC and is believed to influence the balance between the normal NSC pool and its differentiated progeny[19]. Both pathways have been found aberrantly activated in GBM[20-22] and EGFR mutations and over expression are furthermore hallmarks of GBM[23,24]. Recent data moreover suggest that the EGFR and Notch signaling pathways play a role in bCSC growth and survival[25,26]. As such, the EGFR and Notch signaling pathways present interesting targets for bCSC directed therapy for GBM and in the present thesis project we have therefore focused on further dissecting the role of these pathways in bCSC.

2. Background

2.1 Glioblastoma Multiforme

Glioblastoma multiforme (GBM) can develop as secondary GBM from lower grade gliomas or arise as *de novo* primary GBM that accounts for 95% of all GBM[4]. As it is a neurological tumor the symptoms vary greatly, with the most common being paresis and aphasia[27,28], but also include headaches, seizures, cognitive or personality changes, eye weakness and nausea or vomiting[27-29]. However, development of high intracranial pressure is the most threatening feature of GBM[28]. GBM is diagnosed histologically based on the high grade of cytological atypia, anaplasia, mitotic activity, necrosis and microvascular proliferation[30] (Figure 1A and B). The tumor is often located in the cerebral hemispheres with occasionally contralateral invasion and in association to the lateral ventricles and the basal ganglia[28,31,32] (Figure 1C) and due to the very invasive growth pattern total resection is often not possible[33]. The standard treatment today, known as the "Stupp-regime", consists of debulking surgery, followed by radiotherapy (RT) plus concomitant and adjuvant temozolomide (TMZ, Temodal®, an alkylating agent)[34,35]. When RT was introduced to the standard treatment in the late 1970s, the survival of GBM patients improved for the first time, and in 2005, when Stupp published the addition of TMZ, the median survival further increased from 12.1 months to 14.6 months[34] and the five year overall survival from 1.9% to 9.8%[35] (and reviewed in Perry *et al*. (2012)[9]). Despite improvement of survival during the last decades, more than 90% of GBM patients experience relapse[5,36] where the prognosis is even worse (average survival 3-9 month[37]), and a plethora of different targeted therapies have consequently been tested on patients with recurrent GBM. As of today, the most promising results for treatment of recurrent GBM have been obtained with the anti-angiogenic agent bevacizumab (Avastin®)[38] which has been shown to increase progression free survival[37]. However, the effect could only be attributed to responding patients[39] and the overall survival remained almost unaffected[37].

It has been attempted to divide GBM patients into groups depending on how they are expected to benefit from a certain treatment. As an example it has been shown that GBM patients with methylation of the MGMT (*O*-6-methylguanine-DNA methyltransferase) promoter to a higher degree benefit from TMZ treatment and as such have a better prognosis[35,40]. There are thus several indications that GBM patients are a heterogeneous population, and have to be treated accordingly.

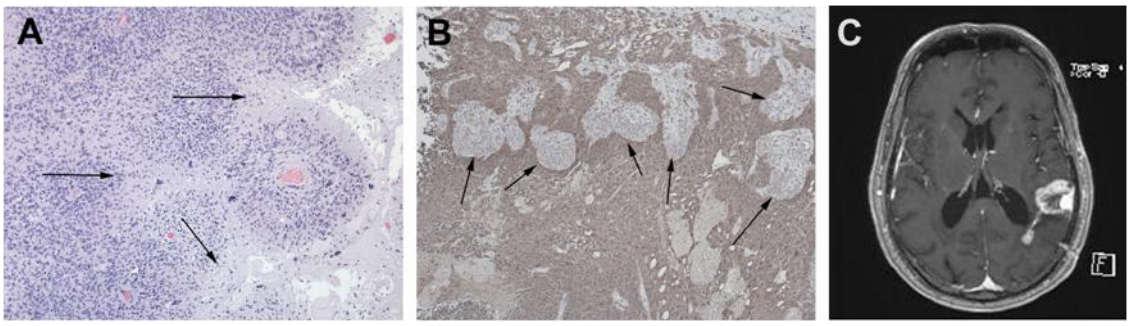

Figure 1: Histology and localization of GBM. Two main histological features are essential for the diagnosis of GBM: Necrosis and excessive vasculature. A) H&E staining showing necrosis with pseudo palisading cells around necrotic foci (see arrows). B) IHC staining of glial fibrillary acidic protein (GFAP) where negative areas represent proliferating endothelial cells (arrows). C) T1 weighted Magnetic Resonance Image (MRI) showing a GBM as a contrast enhanced tumor with necrotic black centre in association with the lateral ventricle. The histological images are kindly lend from Helle Broholm and the MR image is kindly lend from Ulrik Lassen.

As a consequence, several reports are emerging on how to distinguish GBM patient as will be outlined in the following.

2.1.1 GBM sub-types and intratumoral heterogeneity

GBM tumors can be grouped by more markers than MGMT promoter methylation. Over expression of the oncogene *EGFR* and mutations of the tumor suppressor gene *p53* were among the first molecular characteristics that were used to classify GBM, in this case distinguishing between primary and secondary GBM respectively[41]. In fact, *p53* mutations can be tracked from lower grades of gliomas to the progression of secondary GBM[23]. It has subsequently been demonstrated that primary GBM can be further characterized by loss of heterozygosity (LOH) of 10q and mutations in the phosphatase and tensin homolog (*PTEN*) gene[4,42], a negative regulator of the PI3K/AKT pathway downstream of EGFR (see section 2.4.1). It should, however, be emphasized that it is not a black and white picture. E.g. *p53* mutations are also observed in a subset of primary GBM and *PTEN* mutations can be found in some secondary GBM[23].

During the past decade global gene expression analysis has enabled scientists to sub-group GBM with regard to a wide panel of molecular markers. Although there are some discrepancies between the groupings in each study, there are several coincidences. In a study by Verhaak *et al.*[1] 200 GBM samples from The Cancer Genome Atlas (TCGA)[43] were analyzed and the grouping was validated by comparison with previously published gene expression data sets (Phillips *et al*. (2006)[22], Sun *et al*. (2006)[44], Beroukhim *et al*. (2007)[45] and Murat *et al* (2008)[46]). On this basis, Verhaak and colleagues divided GBM into four main sub-types (see also Figure 2): the Classical, the Mesenchymal, the Proneural and the Neural sub-type. The Classical sub-type is characterized by high frequency of chromosomal rearrangements since chromosome (chr.) 7 amplification together with chr. 10 loss were detected in 100% of the classical sub-type tumors. As a result, *EGFR* gene

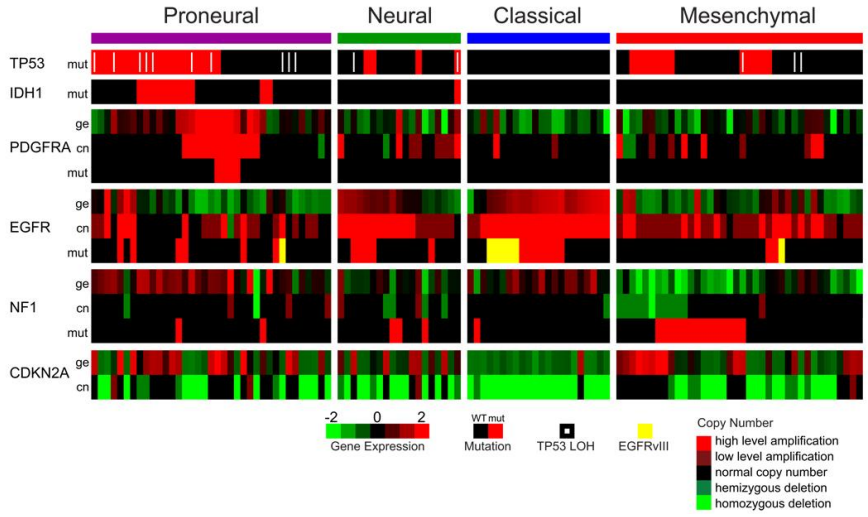

Figure 2: Integrated view of gene expression and genomic alterations across glioblastoma sub-types. Gene expression data (ge) was standardized (mean equal to zero) across 202 dataset. Data are shown for 116 samples with both mutation and copy number data. Mutations (mut) are indicated by a red cell, a white pipe indicates loss of heterozygosity, and a yellow cell indicates the presence of an EGFRvIII mutation. Copy number events (cn) are illustrated by bright green for homozygous deletions, green for hemizygous deletions, black for copy number neutral, red for low level amplification, and bright red for high level amplifications. A black cell indicates no detected alteration. Reprinted with permission from Elsevier publishing (Verhaak *et al*. (2010)[1]).

amplification together with loss of the *CDKN2A* gene (cyclin-dependent kinase inhibitor 2A encoding both the *p16INK4A* and *p14ARF* tumor suppressor genes[47]) was observed in 97% of the classical tumors. This sub-type also demonstrates elevated expression of NES (Nestin, a neural stem cell marker) as well as components of the Notch and Sonic hedgehog (SHH) signaling pathways. The Mesenchymal sub-type is named so due to the high expression of mesenchymal markers such as *CHI3L1* (also known as *YKL40*), *CD44* and *MET* together with the astrocytic marker *MERTK*. Moreover, up-regulation of genes involved in the TNF super family- and NFκB signaling pathways have been observed is this sub-type and, as the author suggests, this might result from the high degree of necrosis and associated infiltrating inflammatory cells seen in this sub-type. Concurrent mutations in the neurofibromin 1 (*NF1*) and *PTEN* genes are also frequently observed. The expression pattern of the Proneural sub-type resembles that of a neural development profile, with the two major alterations being alpha-type platelet derived growth factor receptor (*PDGFRA*) amplification and isocitrate dehydrogenase (*IDH1*) point mutations. *TP53* mutations and LOH were also frequent events. Similar to the classical sub-type, but less frequent, chr. 10 loss paired with chr. 7 amplification were observed (54%). Expression of the oligodendrocytic marker *OLIG2* and other proneural developmental genes such as *DLL3* (encodes Delta-like 3, a Notch ligand, see section 2.5.1), *ASCL1* and *SOX* also characterize the proneural sub-type. Finally, the Neural sub-type is not well defined, but it can be recognized by the expression of neuronal markers. To summarize, Verhaak and collogues concluded that aberrations and gene expression of *EGFR*, *NF1* and *PDGFRA*/*IDH1* each defined the Classical, the Mesenchymal and the Proneural sub-types respectively[1]. Although there was no clear correlation between sub-type and survival, there was a trend towards an increased survival for patients with the Proneural sub-type[1].

By comparing the expression of a pre-defined panel of glioma relevant proteins in 27 GBM surgical specimens and relating them to the TCGA data, Brennan and co-workers defined three groups based on the expression and activation of distinct pathways and named the groups accordingly: the EGFR core, the PDGF core and the NF1 core[48]. The EGFR core showed increased levels of total- and phosphorylated EGFR and was named accordingly. It further resembles the Classical subtype from Verhaak *et al*. as it displayed high levels of the activated intracellular Notch-1 domain (ICN-1), the Notch ligands Jagged-1 (Jag-1) and Delta-like 1 (Dll-1) and the Notch downstream target hairy/enhancer of split-1 (Hes-1). Moreover, genomic analysis revealed that most tumors in this group had chr. 7 gain, *EGFR* amplification and mutation as well as deletion of *Ink4a*/*ARF* and either chr. 10 loss or *PTEN* mutations in all tumors. The PDGF core showed some correlation with the Proneural sub-type from Verhaak *et al*. Compared to the other core-groups it displayed up regulation of PDGFB, phospho-PDGFRβ and phospo-NFKB1. Also an increased level of PTEN was detected as well as increased activation of the Ras pathway as evident by increased levels of phosphor-MEK and -ERK. Moreover it showed expression of the marker OLIG2 which is involved in oligodendrocytic development[49]. None of the tumors in this core group, however, showed amplification of the PDGFR or its ligands. The NF1 core was strongly associated with low levels of NF1 and showed over expression of YKL40 and as such resembles the Mesenchymal sub-type from Verhaak *et al*. It further showed chr. 7 gain, although no EGFR amplification was detected. It should be mentioned that the specimens from Brennan *et al*. included a few Grade III glioma samples, however, the GBM samples were represented in all three core groups.

In an earlier study by Phillips *et al*., which represents one of the four data-sets, utilized in the Verhaak study described above, Grade III and IV gliomas were assigned one of three sub-types: Proneural, Mesenchymal and Proliferative, based on gene expression[22]. The Proneural- and Mesenchymal subtypes were similar to the ones described by Verhaak *et al*., whereas the Proliferative sub-type, when grouped together with some traits from the Mesenchymal sub-type, could be compared to the Verhaak Classical sub-type. (A comparison of the Verhaak, Brennan and Phillips study is presented in Figure 3).

It has been suggested that gene profiling is a superior prognostic marker for malignant gliomas when compared to histological grade or age[50]. Likewise, Phillips *et al.* were able to correlate prognosis with sub-type. The Mesenchymal and Proliferative sub-types were primarily Grade IV gliomas, while the Proneural sub-type comprised gliomas of both Grade III and IV[22]. As the Verhaak study detected frequent *TP53* and *IDH1* mutations in this sub-type[1], and as these features also are common events in secondary GBM[41,51,52], this could indicate that at least some tumors within the Proneural sub-type could represent secondary GBM. And three out of four tumors in the proneural sub-type from the Verhaak study were in fact secondary GBM[1]. In general, Phillip and co-workers stated that tumors with a Proneural signature predicted a better prognosis as compared to tumors with a Mesenchymal or Proliferative signature[22]. Furthermore, it has been suggested that apart from prognosis, sub-type might be correlated to treatment outcome as well (reviewed in Woehrer *et al*. (2013)[2]). It should, however, be considered that in most cases, patient tumor samples used for scientific purposes only represent a small portion of the whole tumor mass, and as GBM tumors are highly heterogeneous, one could speculate that different sub-types might co-exist within the same tumor[46,53]. Moreover, transition between sub-types upon recurrence has been reported[22,46,54]. Still, as the sub-types to some extent can be correlated to prognosis and treatment outcome, they might prove usable in the clinic when stratifying patients to the most optimal treatment. However, until a sub-type specific therapy package is available, full scale sub-typing of GBM patients might be overstated in terms of stratification although individual markers such as MGMT methylation have proven its worth.

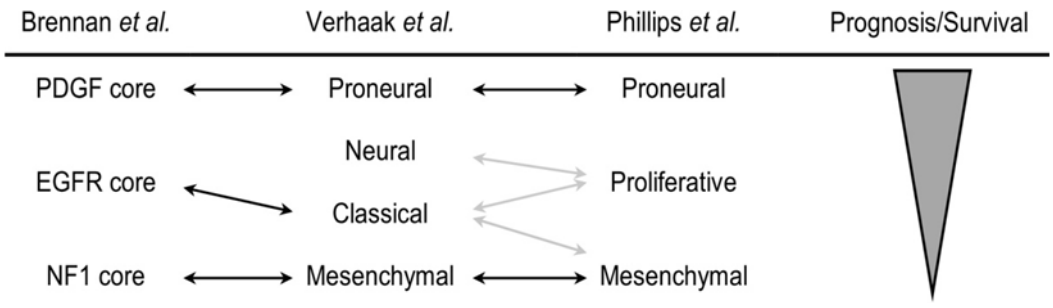

Figure 3: Comparison of sub-type studies. Molecular subtyping of GBM based on gene expression (Verhaak *et al*. and Phillips *et al*.) and protein expression (Brennan *et al*.). Direct comparison across the datasets shows good agreement for Vehaaks and Phillips Proneural sub-type and Brennans PDGF core as well as for the Mesenchymal subtype from Verhaak and Phillips and the NF1 core from Brennan, demonstrated by the black arrows. There is also a good correlation between Brennans EGFR core and Verhaaks classical subtype, black arrows. There is less concordance for Proliferative and Neural/Classical sub-types between Verhaak and Phillips, represented by the grey arrows. Overall, there is an agreement that survival decreases from the Proneural towards the Mesenchymal sub-type. Illustration modified from Woehrer *et al*. (2010)[2].

2.2 Glioblastoma Multiforme models

2.2.1 In vitro models

In vitro cancer models are simple to work with, and offer great insight into cellular pathways and mechanisms involved in cancer cell growth. In addition, they are usually the first step when identifying new therapeutic targets and when testing potential new anti-cancer drugs. Traditional *in vitro* culturing of mammalian cells occurs in the presence of fetal calf serum (FCS) as it contains many important mitogenes and other components that support cell survival and growth. Serum containing cultures have also been widely used for culturing of cancer cells, such as high-grade glioma cells and thus GBM cells. However, when established and cultured in the presence of serum, GBM cells lose important tumor hallmarks, and fail to resemble the original patient tumor, already after a few *in vitro* passages[3,55,56]. As a consequence, commercially available cell lines established and cultured the traditional way are poor experimental models for GBM and have therefore been modified to express GBM hallmarks such as EGFR amplification and mutations. As an example of this is the U87MG cell line that has been modified to contain amplified EGFR and the mutant EGFR variant, EGFRvIII (see section 2.4.2). It should be noticed that the U87MG cell line is of glioma grade III origin (anaplastic astrocytoma, AA), although it has been classified by the American Type Culture Collection[a] (ATCC) as a GBM. As of today, there are no commercially available GBM cell lines with endogenous EGFRvIII expression, and only one GBM cell line, the SKMG3, has been reported to contain endogenous EGFR amplification[57,58].

During the past decade, it has become more common to culture glioma cells in serum-free media, as has been standard when establishing and culturing normal NSC since the mid 1990s. In that context, it has been shown that culture conditions composed of a well defined media with the addition of growth factors such as the epidermal growth factor (EGF) and the basic fibroblastic growth factor (bFGF) support NSC growth and maintainance[59-61], whereas serum addition or growth factor withdrawal induces differentiation of NSC[60,62-64]. In 2006, Lee and colleagues showed that GBM cells cultured as NSC exhibited an expression profile similar to that of the parental patient tumor and normal NSC, while GBM cells established and cultured in the presence of serum showed resemblance to traditional serum cultured, and commercially available GBM cell lines (see Figure 4). Moreover, xenograft tumors derived from NSC cultured GBM cells better recapitulated the pheno- and genotype of the patient tumor, than xenograft tumors derived from serum-containting cultures[3]. The study by Lee and co-workers has subsequently been supported by the demonstration that serum-free GBM cell cultures reflect the cytogenetic of the parental tumor, even after several passages[56]. With the serum-free cell culture media as a base, there have been several attempts to improve the growth of glioma and GBM cells *in vitro*. Above EGF and bFGF also the leukemia inhibitory growth factor (LIF) is believed to act as a mitogen for neural stem- and progenitor cells[65,66], the supplement B27 is thought to improve survival of neural cells[67] and the N2 supplement is by the manufacture recommended for growth of neuroblastomas as well as post-mitotic neurons[b]. As a result, almost every laboratory working with establishing *in vitro* cultures from GBM tumors have more or less developed their own serum-free culturing formula[68].

[a] https://www.atcc.org/
[b] www.invitrogen.com

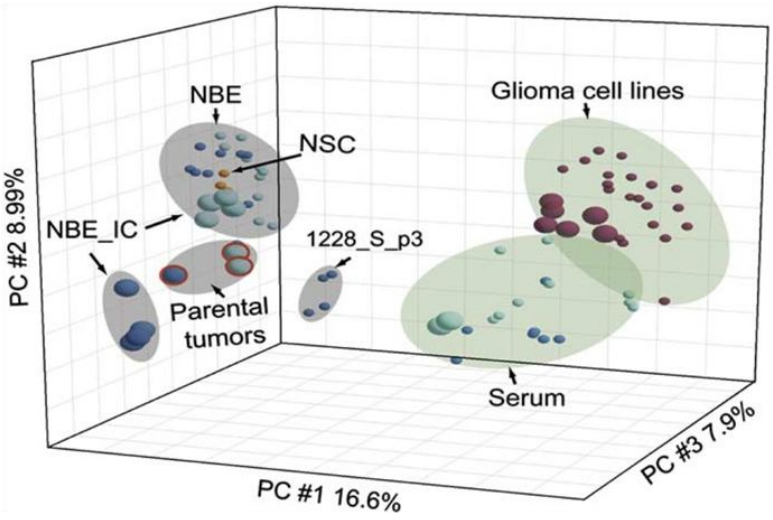

Figure 4: GBM cells cultured in serum-free condition better mimics the gene expression profile of the parental tumor than corresponding serum-cultured GBM cells. Principal component analysis (PCA) of the Lee *et al*. data sets based on global gene expression analysis. Small balls: *in vitro* samples. Large balls: *in vivo* xenograft samples. Colors of balls indicate the origin of samples: Parental patient tumors are marked as balls with red circle, two different tones of blue represent two different parental tumors (1228 and 0308) and thereof derived cultures. Red tone marks commercial GBM cell lines and xenografts (both intracranial and subcutaneous). Yellow balls represent normal NSC samples. NBE_IC indicates intracranial xenograft generated from neurosphere cultures. 1228_S_p3 are 1228 cells at passages 3 in serum containing media. x, y, and z axes represent three major principal components (PC). Note two distinct clusters: one cluster consists of serum-free cultured (NBE) GBM cells and their derivative xenograft tumors, NSCs, and parental patient tumors, whereas the other cluster consists of serum cultured GBM cells, ten commonly used glioma cell lines, and their derivative tumors. Reprinted with permission from Elsevier publishing (Lee *et al*. (2006)[3]).

When cultured during the serum-free NSC conditions, NSC and glioma/GBM cells grow as non-adherent, proliferating cell aggregates called neurospheres, that consist of neurosphere forming cells with multipotent potential as well as more differentiated cells[3,59,60,69-74]. When the neurospheres are dissociated and passaged, the neurosphere forming cells are able to form new neurospheres, which demonstrates their self-renewing ability[72,75], the ability to maintain (or expand) their own population. Upon serum addition or growth factor withdrawal the neurosphere cells become adherent and grow with a more differentiated morphology. The changes in morphology are accompanied by expression of neural differentiation markers, and it has as such been concluded that multipotent cells are present within the neurosphere[3,11,62,63,70-72]. Finally neurosphere cells of both NSC and glioma/GBM origin have been demonstrated to express stem cell markers such as the cell surface glycoprotein CD133[76,77] and the intermediate filament Nestin[69,78]. Because glioma neurosphere forming cells harbors NSC characteristics and moreover have tumorigenic potential[56,70,71,79], they are commonly referred to as brain cancer stem-like cells (bCSC, see section 2.3.2.). The implication of bCSC in GBM tumorigenesis and treatment will be discussed in section 2.3.3.

Although it's many applications, it is important to emphasize that *in vitro* models cannot be representative for all processes within a multi cellular organism, and especially the interaction between a tumor and its surrounding microenvironment require valid *in vivo* models.

2.2.2 In vivo models

In vivo models of tumor growth is pivotal in cancer research as it offers insight into the tumor-host interaction. They are as such essential when studying molecular and genetic events that lead to tumor formation in e.g. the nervous system and serves as indispensable tools when evaluating potential new anti-(brain) cancer treatment strategies. However it should be held in mind that there are some pitfalls when working with animal tumor models: 1) the tumor model may not reflect the biological properties of the patient tumor, 2) the animals used may not display the same pharmacokinetics as humans and 3) the established tumor may not mimic the cellular heterogeneity and properties of the human counterpart (reviewed in Huszthy *et al*. (2012)[80]). There are three main brain tumor animal models: the chemically induced, the genetically engineered and the xenograft model, which will be described below.

The rat is the most popular animal used for chemically induced glioma models. It has been shown that tumors in the rat brain can be induced by administrating methylnitrosourea or ethylnitrosourea compounds[c] either intravenously, orally, locally or transplacentally to the adult or pregnant rat (reviewed in Huszthy *et al*. (2012)[80] and Barth *et al*. (2009)[81]). Chemically induced glioma models may offer insight into chemically initiated human gliomagenesis caused by chemical exposure. However, the exposure time, dose and kinetics of the carcinogenic compound differs between rat and human and moreover no single chemical agent has been implicated in human brain tumor development [82,83]. Furthermore, when intracranial engrafted, rodent glioma cell lines derived from chemically induced tumors show only modest resemblance to human gliomas with regard to morphology and histology[84,85]. As example, no single cell infiltration to the contralateral hemisphere and microvascular abnormalities, characteristic for human GBM, are present in these models, although some invasion can be detected (reviewed in Huszthy *et al*. (2012)[80]).

Increasing knowledge about genomic alterations that possibly play a role in human gliomagenesis has led to generation of genetically engineered glioma mouse models (reviewed in Huszthy *et al*. (2012)[80] and Fomchenko *et al*. (2006)[86]). These models reflect the human tumor histology, biology and etiology[87]. Genetically engineered models are based on either gain or loss of specific genes, in a specific cell type, and at a specific time point in development. This is accomplished by inducing genetic changes in the cell type of interest, e.g. by the cre-lox recombinase or tv-a systems under the influence of a cell specific promoter. One such example is the RCAS/TVA system published by Holland and co-workers[88,89]. Here *RAS* and/or *AKT* were introduced into the viral vector RCAS[d], which subsequently was injected intracranially into the brain of newborn transgenic mice expressing TVA[e] downstream from the *Nestin* promoter. TVA acts as a receptor for the viral vector and as a result, the viral gene construct will only be incorporated into the genome and transcribed in cells where the *Nestin* promoter is active, such as neural progenitor cells, and where TVA is expressed. Using this approach, Holland and colleagues showed that combined activation of RAS and AKT in neural progenitors induced GBM formation in mice[89]. Taken it further, by combining the above described RCAS/TVA system with the cre-lox system Hu *et al.* obtained a similar TVA

[c] Nitrosourea are alkylating compounds with mutagenic and carcinogenic properties. http://www.reference.md/files/D008/mD008770.html

[d] Replication Competent ASLV long terminal repeat with Splice acceptor, derived from the avian sarcoma-leukosis virus-A (ASLV-A).

[e] Member of the low-density-lipoprotein receptor family, encoded by the *tv-a* gene and acts as the receptor for ASLV-A in avian cells.

mouse, although this mouse had *loxP*-sites[f] flanking the *PTEN*-gene. By injecting a RCAS vector containing the Cre recombinase protein gene fused with the green fluorescent protein (GFP), knock out of *PTEN* was obtained in the Nestin expressing cells. This was however, not sufficient to induce lesions, but when combined with RAS activation, GBM was formed[90].

The genetically engineered models have helped scientists to understand the molecular events leading to GBM initiation, progression and metastasis. They are furthermore good models for the tumor-stroma interaction that contribute to malignancy, including angiogenic processes, and as such have expanded our knowledge about the tumor micro-environment and provided insight into the sequence of genomic events that follow a specific genetic alteration. It is however still an open question whether the genetic events that result in tumor formation in experimental animals truly mirror the initiating events in human gliomagenesis (reviewed in Huszthy *et al*. (2012)[80]).

Although both the chemically induced and the genetically engineered glioma animal models provide insight into the events of gliomagenesis, growth and progression as well as the interaction with the surrounding brain parenchyma, these models lack one fundamental feature: the cancer cells are not of human origin. In the xenograft model, human cancer cells are transplanted or grafted onto immunocompromised mice or rats. There are two types of tumor xenografts: the subcutaneous xenograft (SX) were the tumor-cells or tissue are injected or inoculated respectively onto the flanks of the experimental animal, and the orthotopic xenograft (OX), which in the case of brain tumors is established by injecting human brain cancer cells intracranially into the brain of the model animal. Both the SX and the OX model can be established either directly from patient tumor tissue or from *in vitro* cell cultures. The SX model is simple to work with and tumor formation and growth are easy to monitor. However, the OX model is a clinically more relevant model, as the tumor is located in the proper anatomic site, and as such, in the case of gliomas, offers insight into the tumor-brain parenchyma interaction[91] and it has been stated that the micro-environment in the OX model is more comparable with that observed in GBM patients, than the micro-environment in the SX model[92]. Compared to SX, OX are laborious to establish and monitor and require expensive equipment and technical expertise such as MR- and/or CT-PET-scanners. However, the monitoring of OX will on the other hand enable testing of novel imaging methods and different isotopic tracers for PET scans. One major drawback to the xenograft model is the requirement of immunedeficient mice as the immune system is thought to play a significant role in tumor progression and response to therapy[92].

All three types of *in vivo* glioma/GBM models described above, offer possibility for studying signaling pathways and cell-cell- and tumor-stroma interactions important for tumor formation, maintenance and recurrence as well as tumorigenic processes such as angiogenesis and migration/invasion. However, the chemically induced and the genetically engineered models are more relevant for studying events leading to brain tumor initiation and growth, whereas the xenograft model is more suitable for investigating processes involved in tumor maintenance and testing of new therapeutic approaches, as this model is based on naturally transformed human cancer cells. Moreover *in vivo* models are crucial when testing potential new anti-cancer therapies,

[f] Locus of X-over P1, a sequence that serves as binding site for the Cre recombinase protein.

as one can study the effect of the treatment on the whole organism. However species to species differences needs to be taken into account as described above.

2.3 Brain cancer stem-like cells

2.3.1 Development of the CNS

During the fourth week of human embryogenesis, the *craniocaudal* neural tube is formed from invagination of the neural plate consisting of neuroepithelial cells (also designated neuroectodermal cells). This process is known as neurulation and is the first step in development of the CNS comprising the brain and the spinal cord. At this early stage of embryogenesis the vesicles that eventually will give rise to the different regions of the brain are visible. When the neural tube has formed it is lined with proliferative neuroepithelial cells. Most of the cells comprising the future CNS, are produced from these cells present in zones adjacent to the ventricles, namely the ventricular zone (VZ) and the subventricular zone (SVZ) (reviewed in Nowakowski *et al*. (1999)[93]). Thus, the neuroepithelial cells lining the VZ and the SVZ can be considered as multipotent NSC, and are the common precursors for cell types such as neurons, glial and ependymal cells. Importantly, in the adult brain a small SVZ is still detectable and some of its cells continue to proliferate throughout life[94,95] and give rise to neurons and glial cells[73,96].

2.3.2 Definition and origin of bCSC

Growing evidence supports the idea that malignant tumors are initiated and maintained by a population of tumor cells with similar biological properties as normal adult stem cells[17,32,97-99]. The cancer stem cell theory was first demonstrated from research with acute myeloid leukemia (AML)[100] and subsequently cancer stem-like cells have been identified in different solid tumors such as gliomas and GBM. Normal stem cells maintain their population through asymmetric cell division that gives rise to one daughter stem cell (self-renewal) and one cell that displays a more differentiated phenotype, namely a progenitor cell. The progenitor cell will proliferate and give rise to several new identical progenitor cells through symmetric cell division before they become proliferative exhausted and begin to terminally differentiate[75,101-104]. The cancer stem cell hypothesis states that the cancer stem-like cell is able to self-renew as well as give rise to all the differentiated progenies that eventually make up the heterogeneous cell mass of the tumor (see Figure 5). To support this hypothesis, the bCSC have been demonstrated to hold NSC potential as they are able to maintain their own population through self-renewal, able to give rise to cells of the three neural lineages (neurons, oligodendrocytes and astrocytes) and moreover express different NSC markers such as CD133 and Nestin, as mentioned above (section 2.2.1). Besides from their NSC-like characteristics, bCSC are

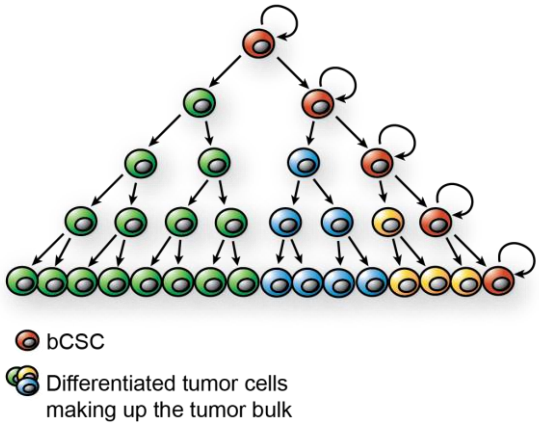

Figure 5: The brain tumor cell hierarchy. In the cancer stem cell model, the bCSC (red) have the ability to maintain its population through self-renewal (circular arrow) and give rise to more differentiated tumor cells (green, yellow, blue), that make up the majority of the tumor bulk.

tumorigenic and able to form xenograft tumors resembling the parental tumor when transplanted onto immunocompromised mice[3,79,105-109].

It has, however, not yet been established whether bCSC originate from normal undifferentiated cells such as NSC and progenitor cells or from de-differentiation of more mature tumor cells, and as such, are a result of tumor progression instead of the initiator. As described in section 2.1, human brain tumors are known to frequently arise near the SVZ and other neurogenic areas of the brain and it has been reported that cells in these areas, including NSC, are more sensitive to transformation than differentiated cells and hence more likely to form tumors upon mutagenic exposure or oncogene activation[89,90,110-112]. As an example, using the RCAS/TVA model outlined above, Holland and co-workers showed that when the *TVA* gene expression was under control of the astrocytic glial fibrillary acidic protein (*GFAP*) promoter no tumor formation was observed upon transfection with RCAS-AKT or -RAS, while gliomas with histological features of GBM arose when the *TVA* gene was downstream from the Nestin promoter and hence specifically expressed in the NSC population[89]. In addition, *in vitro* spontaneous transformation of non-tumorigenic low-grade glioma cells and SVZ derived NSC into highly tumorigenic immortal cell lines resembling bCSC has also been reported[97,98,113]. So even though the cancer stem cell hypothesis does not require bCSC to evolve from normal NSC, there are several indications that this could be the case. Nevertheless, the hypothesis that bCSC represent a highly potential therapeutic target for novel anti-GBM treatment is increasingly being accepted.

2.3.3 Role in GBM

It has been suggested that present glioma treatment fails because it only kills the bulk of the tumor, whereas the tumor initiating bCSC escape and are able to regenerate the tumor and cause relapse, as reviewed in Massard *et al*. (2006)[114]. One reason for the inadequate effect of treatment is ascribed to the fact that most cytotoxic treatment is aimed at fast dividing cells (corresponding to tumor progenitor cells[115]), while the bCSC are spared as they are normally quiescent[116,117] or slowly cycling[118]. Furthermore, CD133 positive (CD133+) cells have been demonstrated to have an elevated expression of multi-drug resistance genes, DNA mismatch repair genes and genes inhibiting apoptosis [14,119] as well as decreased radio-sensitivity[120] as compared to CD133 negative (CD133-) cells. In fact, in response to radiation, bCSC have been shown to activate the DNA damage checkpoint response and increase their DNA damage repair activity, thus decreasing apoptosis[13]. Together these findings support the idea that bCSC contribute to chemo- and radio-resistance. Moreover, recurrent GBM have been reported to have an increased level of CD133+ cells as compared to newly diagnosed GBM tumors[14], and cell cultures have been found to be enriched with CD133+ cells after radiation[13], suggesting that traditional anti-GBM treatment selects for a bCSC population and it could be speculated that bCSC are responsible for recurrence of the tumor after therapy with increased aggression. It should however be held in mind that CD133 may not serve as a distinct bCSC marker as also CD133- glioma/GBM neurosphere cells are able to form xenograft tumors[121-123].

In the adult mammalian brain, NSC have been demonstrated to produce progenitor cells that migrate away from the stem cell niche, along well-defined streams, to undergo terminal differentiation in a different CNS area, both under normal conditions and as a response to tissue

injury[102,124]. This ability seems to be conserved in the bCSC progeny as they are able to migrate unorganized throughout the brain parenchyma and initiate tumor formation in adjacent brain regions[99]. It is thus likely that bCSC are present in areas of great invasiveness and were the resection is not optimal. As a consequence some bCSC will remain in the brain after surgery were they are able to avoid chemo- and radiation therapy and will continue to produce migrating progenitors that repopulate the tumor and ultimately cause relapse. In fact, recurrent gliomas can be found at the initial tumor bulk site, or anywhere ells throughout the brain parenchyma, including the ventricles[125-128].

Finally, bCSC might also be involved in tumor angiogenesis [15,44] possibly by producing the pro-angiogenic vascular endothelial growth factor (VEGF). Angiogenesis is pivotal for tumors to grow more than a couple of millimeters in diameter. It is initiated by hypoxia that also is known to support survival and proliferation of NSC and progenitor cells and it has been shown that low O_2 tension inhibit differentiation and thus maintain stem cell characteristics[129-131]. Several studies suggest that hypoxia has a crucial role in glioma growth and tumorigenecity[132-135], and that the mechanism behind is shared between NCS and bCSC (reviewed in Diabiri *et al*. (2008)[136]). In line with this, Bao *et al*.[15] detected massive angiogenesis, necrosis and haemorrhage when transplanting bCSC into severe combined immunodeficiency (SCID) mice, in contrast to when transplanting non-bCSC. They further measured the expression of a panel of angiogenic factors and found that bCSC consistently secreted an elevated level of VEGF. By culturing endothelial cells (EC) in bCSC conditioned media, they found significant increase in EC migration and tube formation when compared to non-bCSC conditioned media. Taken together, bCSC might prove crucial for GBM angiogenesis possible by responding to low oxygen levels by secreting VEGF and thus increasing angiogenesis, which would merely substantiate the need for developing bCSC targeted anti-GBM therapy.

2.3.4 Implication in treatment of GBM

If bCSC are responsible for tumor initiation, progression, chemo- and radio resistance and hence tumor relapse they might be thought of as the "mother population" of the tumor and as such serve as a potential powerful new target for GBM treatment. The hypothesis is that if the tumor bulk needs to be maintained by generation of cancer cells from the bCSC population, elimination of the bCSC will lead to shrinkage and ultimately eradication of the tumor. In addtition, if the bCSC are eliminated together with traditional chemo- and radiation therapy that targets the bulk of the tumor, there will be no regeneration of more differentiated tumor cells that constitutes the tumor bulk and, moreover, the risk of dedifferentiation of the more mature tumor cells into new bCSC, as described in 2.3.2, is minimized (see Figure 6.). Targeting the bCSC could either lead to killing the cells or prevent them from regenerate the heterogenic tumor mass. The latter could be accomplished by forcing the bCSC to differentiate. Differentiating therapy has been utilized for treatment of acute promyelocytic leukemia (APL), in which poorly differentiated leukemia cells populate the bone marrow, hindering the production of normal blood cells. Using *all*-trans-retinoic acid (RA) the immature cancer cells are forced to differentiate and thereby lose their malignant potential[114,137]. In the study be Lee and co-workers (see section 2.2.1), they demonstrate that when GBM neurosphere cells are cultured in the presence of RA they express differentiation markers at similar level as

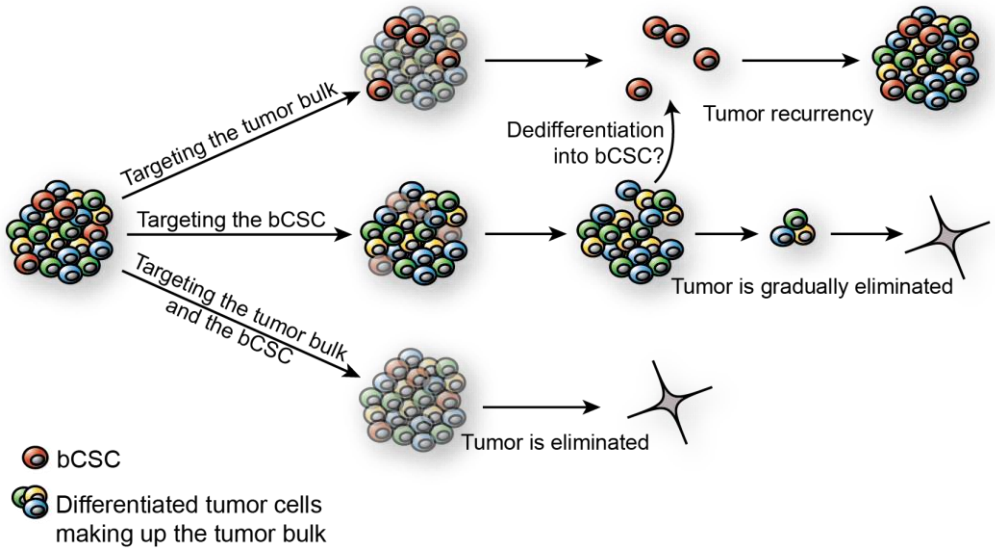

Figure 6: Targeting the bCSC in GBM therapy. According to the bCSC hypothesis, traditional therapy (top row) will only hit the tumor bulk leaving the bCSC to re-generate the tumor and cause relapse. Targeting the bCSC (middle row) will lead to gradual elimination of the tumor but will not eliminate the risk of dedifferentiation of more mature tumor cells into new bCSC. Therapeutic targeting of both the bCSC population and the more differentiated tumor bulk cells (bottom row) is thus important in order to fully eliminate the tumor and prevent relapse.

GBM cells grown in serum-containing media[3]. It has likewise been demonstrated that U87 derived neurosphere cultures differentiate when treated with low concentrations of RA[138]. It might as such be feasible, using this or similar differentiation approaches, to force the bCSC population to differentiate and hinder them from (re-)populate the tumor bulk.

Another approach to target the bCSC could be through signaling pathways known to be important for the maintenance of the normal NSC population such as the SHH, Wnt, TGF-β, BMP, EGFR and Notch pathways, of which the latter two are described below.

2.4 EGFR signaling

Growth factors and their receptors play a central role in the regulation of a number of cellular processes including cell survival, metabolism, proliferation, differentiation and migration. Among the best described growth factor regulated pathways are those mediated by receptor tyrosine kinases (RTKs) which are multifunctional proteins with comparable structural features. These include an extracellular ligand binding domain, composed of four sub-domains (I-IV), that is usually glycosylated, a single transmembrane helix domain, and an intracellular domain containing a conserved protein tyrosine kinase domain and regulatory sequences that are subjected to auto-phosphorylation and phosphorylation (reviewed in Hunter *et al*. (1998)[139], Hubbard *et al*. (1998)[140], Schlessinger *et al*. (2000)[141] and Zandi *et al*. (2007)[20]). The first RTK to be discovered was the 170 kDa EGFR[20,142] (ErbB1/HER1, see Figure 7) that belongs to the ErbB/HER (avian homolog erythroblastic leukemia viral (v-erb-b) oncogene, human homolog named HER[143]) family of ligand activated tyrosine kinase receptors which also comprise ErbB2 (HER2/Neu), ErbB3 (HER3) and ErbB4 (HER4) (reviewed in Holbro *et al*. (2003)[144], de Bono *et al*. (2002)[145] and Burgess *et al* (2008)[146]).

In the normal brain, EGFR is expressed in neurogenic areas where also NSC are abundant[18,147,148] and it has been shown that EGFR is involved in regulation of the developmental and adult stage of NSC proliferation, migration and differentiation[18,148-153]. Both NSC and bCSC proliferate *in vitro* in response to EGF and other EGFR-ligands [3,61] and recent studies have moreover demonstrated that a bCSC population can be isolated based on the expression of EGFR[154] and that GBM neurosphere cultures are sensitive to inhibition of EGFR signaling[25,155,156]. Furthermore, EGFR expression together with bCSC signature has been associated with chemo- and radiation resistance[46].

2.4.1 The pathway

It has been proposed that RTKs, like the EGFR exist as monomers in the cell membrane perhaps in equilibrium with partly dimerized and partly activated receptors[20,157-160] (Figure 7). Ligand binding (i.e. EGF, transforming growth factor (TGF) -α, amphiregulin, betacellulin, epiregulin, heparin binding EGF-like growth factor (HB-EGF) (reviewed in [146,161])) induces conformational change and receptor dimerization and/or stabilization of the already existing dimers[157,159,160,162]. Dimerization can occur as homodimerization in where i.e. EGFR dimerizes with another EGFR, or heterodimerization where i.e. EGFR dimerizes with another ErbB/HER family member[162]. Ultimately this will result in activation of intrinsic tyrosine kinases in the cytosolic domain of the receptor. When no ligand is bound, the tyrosine kinase domain is intrinsically inhibited[161]. But upon ligand binding and conformational change the tyrosine kinase is activated and catalyzes the transfer of a phosphatase group from donor adenosine triphospate (ATP) to an acceptor hydroxyl group of tyrosine residues residing near the catalytic site on the dimer neighbor[139,141]. This tyrosine phosphorylation subsequently leads to phosporylation of additional tyrosine residues in the tail of the cytosolic EGFR domains, with the two cytoplasmic domains acting simply as substrate and

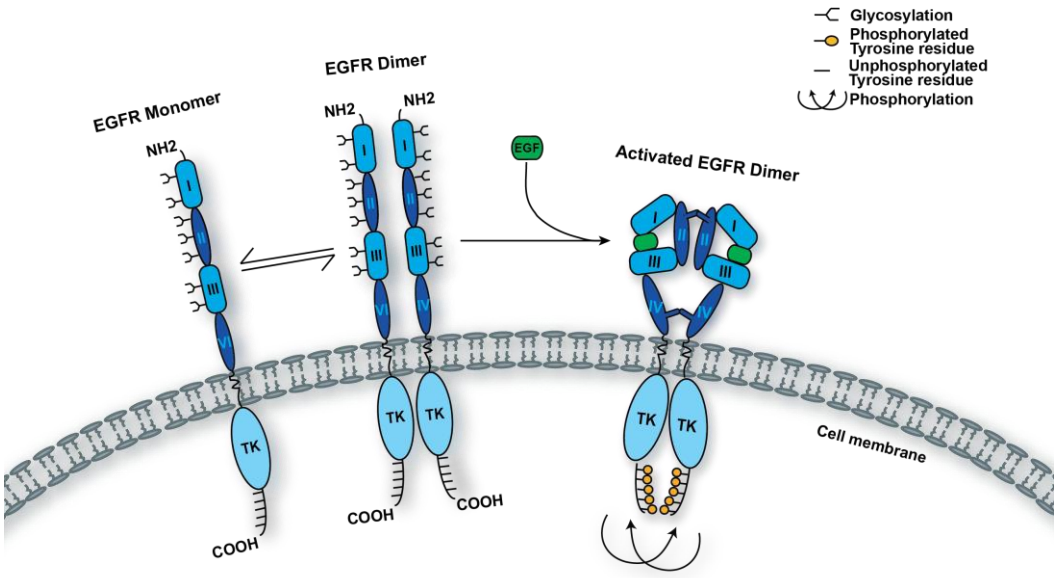

Figure 7: Schematic structure of EGFR. The EGFR receptor is composed of three main domains: an extracellular domain, a transmembrane domain and an intracellular domain. The extracellular ligand binding domain is made up of four sub-domains, designated I-IV and the intracellular domain holds the tyrosine kinase. It is proposed that the monomer and dimer receptor exist in equilibrium and ligand binding induces conformational change of the receptor and stabilization of the dimer leading to phosporylation and activation of the intracellular domain that now can serve as docking site for downstream signaling molecules. See text for details.

enzyme for one another[139-141,159,161] (Figure 7). These phosphorylated tyrosine residues then serve as docking sites for adapter and signaling molecules leading to the activation of several signaling pathways downstream from the receptor[159,162,163]. For an overview, see figure 8.

One of the best characterized EGFR effector pathways is the mitogen activated protein kinase (MAPK, also named extracellular signal-regulated kinase, ERK) signaling cascade, composed of, among others, the mediators RAS (GTPase), RAF (serine/threonine kinase), MEK (MAPK/ERK kinase), and ERK. Phosphorylated tyrosine residues within the EGFR cytosolic domain recruit the growth factor receptor-bound protein 2 (Grb2) which facilitates the binding of the guanidine exchange factor Son of Sevenless (SOS) that exchange the RAS-bound guanine diphosphate (GDP) for guanine triphosphate (GTP) and thus activate the G-protein RAS. RAS then phosphorylates and activates RAF that in turn phosphorylates and activates MEK. Finally MEK phosphorylates and

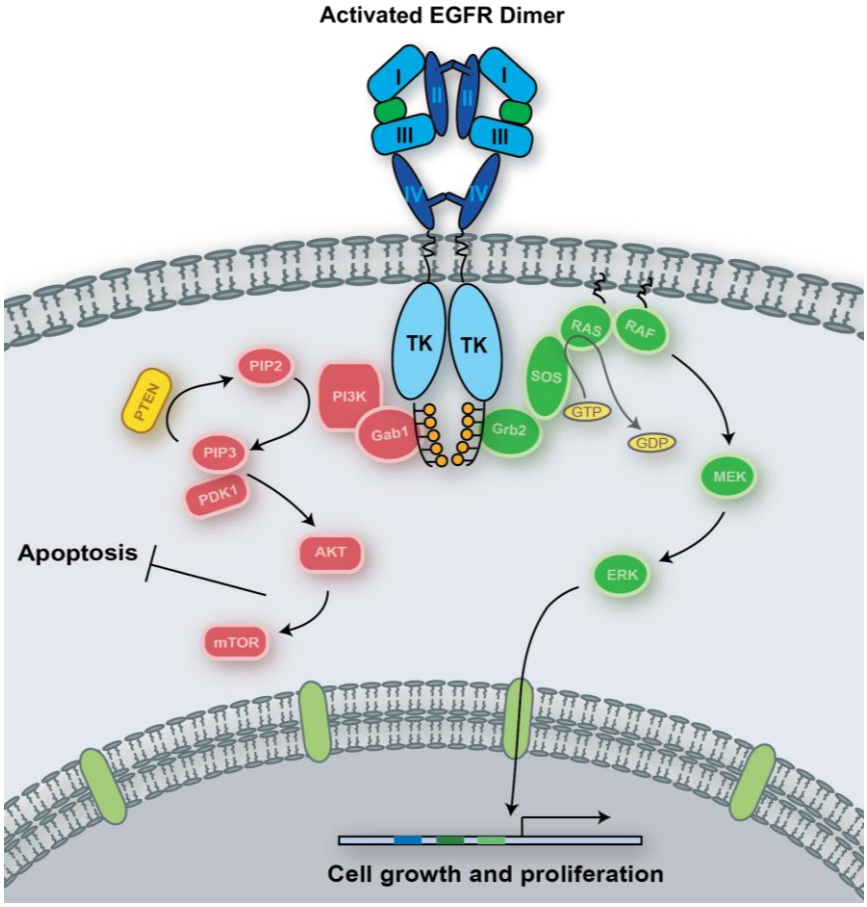

Figure 8: Downstream signaling pathways induced by EGFR activation. Schematic overview of two of the most prominent signaling cascades activated by EGFR. RAS/RAF/MEK/ERK pathway (green) and the PI3-K/AKT pathway (red). Both signaling pathways can be thought of as phosporylation cascades initiated by the docking of an adaptor molecule to the phosporylated tyrosine residues in the intracellular receptor domain and subsequently one intracellular signaling molecule phosporylating the next culminating with regulation of the activity of target molecules and/or alteration of gene expression. EGFR signaling has a wide range of effects including cell survival by evasion of apoptosis and cell growth and proliferation. AKT: Protein kinase B, ERK: Extracellular regulated kinase, Gab1: Grb2-associated protein 1, Grb2: growth factor receptor-bound protein 2, MEK: MAPK/ERK kinase, mTOR: mammalian target of rapamycin, PDK1: 3-phosphoinositide dependent protein kinase-1, PI3K: phosphatidylinositol 3-kinase, PIP2: phosphatidylinositol (4,5)-bisphosphate, PIP3: phosphatidylinositol (3,4,5)-trisphosphate, PTEN: Phosphatase and Tensin homolog, RAF: serine/threonine kinase, RAS: G-protein, SOS: son of sevenless, TK: Tyrosine kinase. See text for details.

activates ERK/MAPK that subsequently translocates to the nucleus were it phosphorylates and activates many target proteins including nuclear transcription factors that subsequently activate transcription of target genes[163-165]. The biological effects of the MAPK pathway are many, but mainly they lead to cell growth and proliferation (Figure 8, green pathway).
Another pathway downstream of EGFR involves the phosphatidylinositol 3-kinase (PI3-K), which binds to the phosphorylated EGFR through another adapter protein, the Grb2-associated protein 1 (Gab1). PI3-K phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP-2) to form phosphatidylinositol (3,4,5)-trisphosphate (PIP-3) that in collaboration with PDK1 (3-phosphoinositide dependent protein kinase-1) recruits and activate AKT ("AK" was a temporary classification name for a mouse strain developing spontaneous thymic lymphomas. "T" stands for transforming, also known as protein kinase B (PKB)), by phosphorylation. The PI3-K/AKT pathway is regulated by the tumor suppressor PTEN, which antagonizes the action of the PI3-K, by dephosphorylation of PIP3 to PIP2[163-165]. Activated AKT has many downstream targets such as the mammalian target of rapamycin (mTOR, a serine/threonine kinase), and has been shown to promote cell survival by inhibiting apoptosis (Figure 8, red pathway).

2.4.2 Mutations of EGFR and downstream mediators

EGFR has frequently been implicated in various forms of human cancers of epithelial origin including brain, lung, breast, head and neck, gastric, colorectal, esophageal, prostate, bladder, renal, pancreatic, and ovarian cancers[166]. The mechanisms by which EGFR becomes oncogenic are several and include autocrine growth factor loops, over expression of EGFR, and gene mutations giving rise to constitutively active variants of EGFR[20,161,166,167]. The events leading to over expression, as seen in approximately 50-60% of GBM (reviewed in[9,168]), can be increased activity of the EGFR promoter, amplification of the EGFR gene or deregulation at the translational and post-translational level[20]. EGFR mutations are present in 40-50% of GBM. At least nine mutation variants have been identified, of which with the constitutively activated EGFRvIII is the most common[20,164,169,170]. EGFRvIII is a result of an in-frame deletion of 801 base pairs, corresponding to exons 2-7 in the EGFR gene[171]. The deletion eliminates 267 amino acids (amino acid 6-273) from the extracellular domain and results in a 145 kDa truncated receptor with a distorted ligand binding area[143,169,171]. As a result, EGFRvIII is unable to bind any EGFR ligands, but is still properly embedded in the membrane were it exhibits a constitutively low activation of the tyrosine kinase and thus is able to activate downstream signaling pathways[161,172-174] (Figure 9). In that context it has been shown that EGFRvIII has a higher signaling trough the PI3-K/AKT pathway, than wild type EGFR[173,175]. Despite the lack of ligand binding, EGFRvIII is able to form not only homodimers with itself, but also heterodimers when co-expressed with EGFR and interestingly EGFRvIII expression almost exclusively occurs in tumors with EGFR amplification (~40% of tumors with amplified EGFR[176], while EGFR amplification often is observed alone[55,171,176]).
Downstream from EGFR, constitutively active RAS mutations have been reported in a variety of tumors but are rare in GBM. However, increased RAS-activity is a frequent phenomenon in GBM possibly due to increased activation of the upstream RTK[177-179]. In addition, several studies have shown mutations in the AKT-interacting regions of PI3-K, which could contribute to increased activation of the PI3K/AKT pathway in GBM[180,181]. The same is the case with inactivation of PTEN

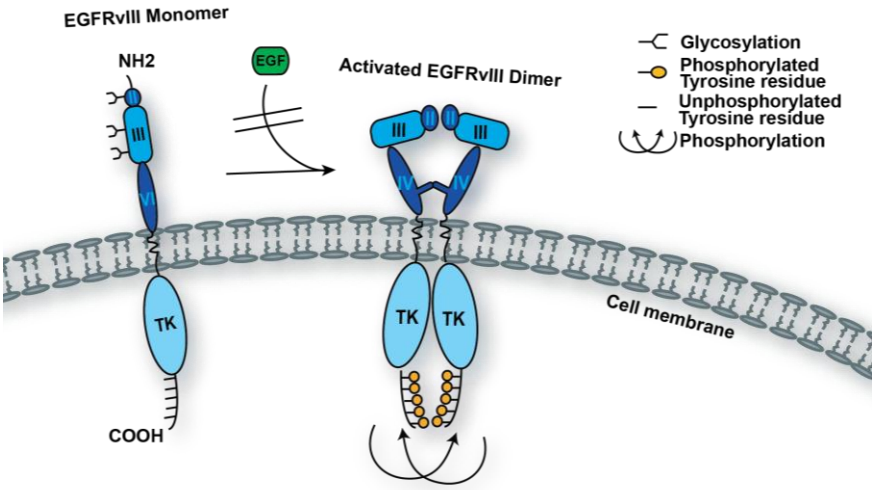

Figure 9: Schematic structure of EGFRvIII. The mutated receptor EGFRvIII is a result of an in-frame deletion of exons 2-7 and therefore it lacks amino acid 6-273 corresponding to sub-domain I and most of sub-domain II. Thus, EGFRvIII is unable to bind a ligand but is located in the cell membrane were it demonstrates a low but constitutive activation.

due to *PTEN* mutations or loss (see section 2.1.1), which on top of increased EGFR signaling, contributes the abnormal high activity of the PI3K/AKT pathway, often seen in primary GBM[42,170,182] and has been correlated to the dismal prognosis of patients with GBM[23,183]. Taken together, multiple alterations of the EGFR signaling pathway have been reported in GBM and other cancer types, and this pathway thus serves as a potential target for anti-GBM therapy.

2.4.3 Inhibitors and clinical implications for GBM

As outlined above, there exist several indications that alteration of EGFR signaling is involved in the pathogenesis of GBM. This is further supported by observations that over activation and/or mutation of the EGFR pathway results in cell proliferation, increased tumor invasiveness, motility and angiogenesis[170,172,177] and has led to an extensive interest in the search for of therapeutic strategies targeting EGFR. The clinically most advanced strategies for inhibition of EGFR signaling are small molecule inhibitors directly targeting the highly conserved tyrosine kinase domain in the intracellular region (tyrosine kinase inhibitors, TKI) and the use of monoclonal antibodies (mAb) targeting the extracellular ligand binding domain. But also inhibition of downstream mediators, ligand- and mAb conjugated toxins and RNA-based therapies are being tested (reviewed in Nedergaard *et al*. (2012)[161] and Karpel-Massler *et al*. (2009)[184]).

TKIs are ATP-competitive inhibitors of the tyrosine kinase located at the intracellular part of the EGFR that result in inhibition of EGFR phosphorylation. Gefitinib (Iressa®) and erlotinib (Tarceva®) are examples of first generation TKIs that have been used in phase I and phase II clinical trials for high grade gliomas, either as monotherapies or in combination with conventional chemotherapy. Although there were some indications of a modest effect of the EGFR targeting TKI, the results from these studies were conflicting (reviewed in Karpel-Massler *et al*. (2009)[184]). Moreover, contraindicating results regarding the possibility to predict the response to TKIs by EGFR expression level exist. E.g. response to gefitinib could not be predicted by EGFR expression level or mutation[185], while a study from our laboratory has demonstrated that EGFRvIII positive GBM cells showed insensitivity towards gefitinib compared to wild-type EGFR cells[186] and others have correlated EGFR amplification to erlotinib response[187,188].

As mAbs usually are generated in mice, the issue of an immune response in patients upon repeated administration has led to the development of chimeric mAbs for therapy purposes. A chimeric mAb is a fusion between the antigen recognizing variable part of the murine derived mAB and the constant part of a human antibody[189]. Cetuximab (Erbitux®) is a chimeric mAb of the IgG1 type that binds to the extracellular domain of the EGFR with high affinity and competes for ligand binding as well as hindering the formation of EGFR dimers[190,191]. In addition, Cetuximab also recognizes EGFRvIII despite the lack of the ligand binding domain and for both EGFR variants, cetuximab inhibits phosporylation as well as induces down regulation of the receptors on the cell surface by promoting internalization[190,192-194]. Only a limited number of studies have tested cetuximab for high grade glioma patients. Belda-Iniesta and co-workers showed some durable responses when using cetuximab in three heavily pre-treated patients with recurrent GBM. Compared to the median survival of patients with recurrent GBM, which is three to nine months when treated with traditional therapeutic agents[195,196], these three patients remained clinically and radiologically stable for 14, 13, and 11 months, respectively. In a phase II study from Copenhagen by Hasselbalch and colleagues, the combination of cetuximab, bevacizumab (VEGF inhibitor) and irinotecan (topoisomerase I inhibitor) resulted in durable response, including two complete responses. However, the response rate and survival data did not seem superior to treatment without cetuximab, and the authors concluded that the combination of cetuximab, bevacizumab and irinotican could not be recommended for treatment of recurrent GBM[197]. Data from *in vitro* and *in vivo* preclinical studies with cetuximab using glioma cell lines with EGFR over expression and/or mutations show contradictive results. Some studies show reduced cell viability upon treatment[198-200], whereas others have demonstrated that cetuximab was insufficient in inhibiting glioma cell growth most likely due to maintained signaling downstream from EGFR[201]. In that regard it has been suggested that increased AKT activity predicts decreased response to the TKI erlotinib, as low levels of phosphor-AKT was seen in patients responding to treatment[187,188]. These studies also suggest EGFR over-expression and amplification as a positive determinant for erlotinib treatment. Also the EGFR mutational status has been suggested to predict the outcome of cetuximab in combination with TMZ and/or RT, as xenografts bearing EGFRvIII positive tumors were more sensitive to the treatment than xenografts expressing wild-type EGFR[202]. In consensus, by analyzing 500 epithelial derived cancer cell lines it was found that cells sensitive to EGFR or HER2 inhibitors were characterized by activating mutations of the target gene[203].

The inconsistent results from studies targeting EGFR signaling, both by means of TKIs and mAbs, in GBM leads to speculations whether GBM patients could be stratified to an EGFR targeted anti-GBM treatment, and the effect of EGFR inhibition in GBM still needs to be clarified. There are as such, ongoing *in vitro* and *in vivo* studies using TKIs or mAbs looking at the various cellular and molecular effects of EGFR inhibition. With a deeper understanding of how and when EGFR inhibition has an effect in combination with the development of sub-type grouping of GBM tumors, stratification of patients that will benefit from an EGFR targeted anti-GBM treatment might be feasible.

2.5 Notch signaling

The Notch gene locus was first described in 1917 in *Drosophila Melanogaster (D. Melanogaster)* where the mutant allele gave rise to flies with "notched" wings[204]. The Notch signaling pathway is evolutionary conserved and plays a fundamental role in several developmental processes all the way from the four cell stage, through formation of the three germ layers to development of adult organ systems such as the central nervous system[205]. Notch signaling has a diverse impact on several cellular pathways and functions depending on the cellular context, the activating ligand as well as intervention from additional signaling pathways. In the brain, Notch signaling is believed to influence the balance between the NSC pool and its differentiating progeny both during development and in the adult (reviewed in Androutsellis-Theotokis *et al*. (2006)[206] and Imayoshi *et al*. (2010)[207]). Clinically, GBM are often located in close association to neurogenic areas of the brain, such as the SVZ[28,31,32] as dexribed in section 2.1, where NSC are abundant[69] and as such, it is no surprise that that Notch pathway components often are found aberrantly expressed in GBM and thereof derived neurosphere cultures[3,11,105,208-211], indicating a role for Notch signaling in bCSC.

2.5.1 The canonical pathway

The Notch receptors (Notch 1-4 in mammals) are synthesized as 300 kDa proteins, which are cleaved by the furin-like convertase (Figure 10, S1) in the *trans*-Golgi apparatus of the secretory pathway, generating a 180 kDa extracellular ligand binding domain and a 120 kDa transmembrane/intracellular domain[205]. Remaining non-covalently bound to each other, the domains are embedded into the plasma membrane as heterodimeric receptors with the transmembrane domain extending into the cytoplasm[212,213]. Activation of the Notch receptors is initiated through juxtacrine binding of a ligand (Delta-like (Dll) 1, 3-4 and Jagged 1-2 in mammals) located on a neighboring cell. Through a conformational change the receptor is then sensitized to two additional proteolytic events (Figure 10, S2 and S3), mediated by members of the ADAM and γ-secretase families of proteases respectively[214,215]. The second cleavage (S2) is thought to be crucial for removing inhibitory components of the extracellular domain and exposure of the third and activating cleavage site present in the transmembrane domain. The third cleavage (S3) results in activation of the receptor and release of the intracellular Notch domain (ICN). ICN is translocated to the nucleus where it binds to the CSL (CBF, Suppressor of Hairless, LAG-1; also referred to as RBP-Jκ) transcription factor[214]. In the absence of ICN, CSL binds to at least two co-repressor complexes (CoR): the SMRT/Nco-R/histone deacetylase 1 (HDAC1) complex and the CIR/HDAC2/SAP30 complex (reviewed in Mumm *et al*. (2000)[216]). The association between ICN and CSL mediates the exchange of the CoR with a co-activator complex (CoA)[217], converting CSL from being a transcriptional repressor to an activator that initiates transcription of Notch target genes. Besides CSL and ICN, the transcriptional activator complex is thought to be composed of the co-activators Mastermind-like (MAML-1, 2 and 3) and the histone acetyltransferase (HAT) p300/CBP [216,218,219] and possibly the HATs pCAF and GCN5[216,219]. Many target genes of Notch signaling have been shown to contain DNA binding sites for CSL (GTGGGAA[220,221]) in their promoter regions[222,223] and CSL is thought to mediate the majority of the downstream effects of the Notch pathway, although CSL independent gene expression has been reported[224,225].

Members of the Hairy/enhancer of split (Hes-1-7) family of basic helix-loop-helix (bHLH) transcriptional repressors are some of the best characterized effectors of Notch activation. They are known to repress the transcription of, among others, the pro-neuronal bHLH protein mammalian achaete-scute homologue 1 (Mash-1, Hash-1 in humans)[226,227]. In addition to Hes also the bHLH transcriptional repressor hairy/enhancer of split related with YRPW motif protein (Hey-1, -2, L) [222], which functions similar to Hes, the glial fibrillary acidic protein (GFAP) [223] that, besides from being an astrocytic marker, also is expressed in postnatal NSC[73,228,229], the NSC marker Nestin [78,230] and the cell cycle regulators p21 and Cyclin D1[231-233], have been suggested as Notch transcriptional targets and the list is still growing.

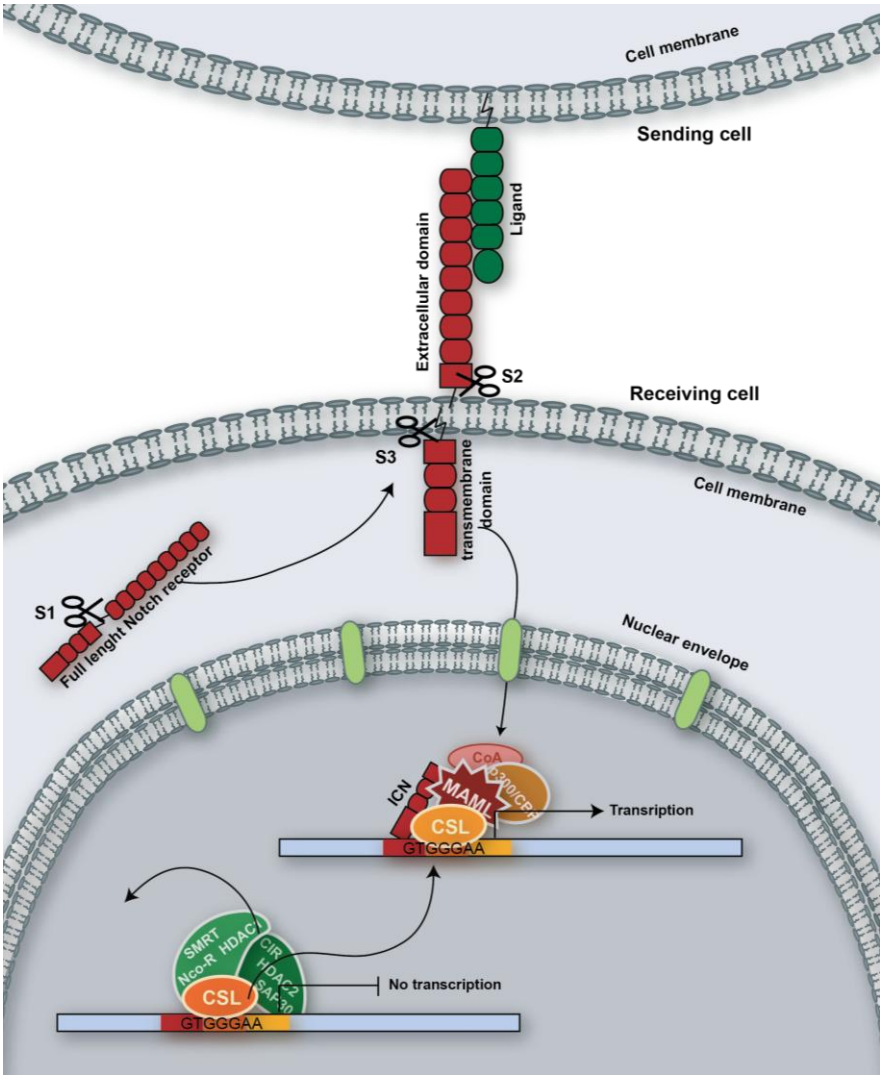

Figure 10: Schematic overview of Notch receptor activation and the down-stream signaling pathway. The Notch receptors are synthesized as large proteins that are cleaved (S1) and inserted in the membrane as heterodimers. Interaction of the Notch receptor with one of its ligands, leads to two consecutive cleavages (S2 and S3) and ultimately release of the ICN. In the nucleus, ICN associates with the transcription factor CSL, which displaces a co-repressor complex (the SMRT/Nco-R/HDAC1 complex and the CIR/HDAC2/SAP30 complex) and recruits a co-activator complex composed of, among others MAML and p300/CBP, leading to transcription of target genes. S1 is mediated by furin-like. S2 is mediated by ADAM. S3 is mediated by γ-secretase. CoA: Co-activator, CSL: CBF - Suppressor of Hairless - LAG-1, HDAC: histone deacethylase, ICN: intracellular Notch domain, MAML: Mastermind-like, p300/CBP: histone acetyltransferase (HAT). See text for further details.

2.5.2 The role of Notch in normal development

Notch signaling during brain development

The importance of Notch signaling in restricting cell fate decisions throughout neurogenesis was initially described by loss-of-function mutations in *D. Melanogaster* generating a "neurogenic" phenotype in which excessive neuronal differentiation was observed at the expense of epidermis[234]. Notch has been associated with undifferentiated cells of the embryonic CNS whereas its expression is reduced in the adult[235]. In the CNS, Notch signaling is thought to maintain a pool of undifferentiated progenitors by inhibiting neuronal commitment and thereby differentiation into neurons[212]. As such, Notch is expressed in proliferating cells of the neural tube, whereas Delta expression is detected in cells eventually becoming neurons[236-238]. Studies in mice, chicken and frogs have shown that *Notch-1* and *RBP-Jκ* mutants, which die early during embryogenesis, lack neuroblasts and show premature neuronal differentiation of the neural tube, indicating a role for active Notch signaling in preventing neurogenesis[239-242]. In opposite, gain-of-function studies have demonstrated that forced Notch signaling can prevent progenitors from undergoing neurogenesis[240,241]. Furthermore, the Notch target Hes-1 has been associated with neuronal precursor cells whereas its expression is absent in mature neurons [243]. The knowledge of Hes-1 in neuronal differentiation came from over expression and deletion studies in mice showing that persistent Hes-1 expression inhibits neuronal differentiation and migration of neurons from the VZ whereas lack of Hes-1 leads to premature neuronal differentiation and open brain anencephaly as a result of failure to close the neural tube[226,243,244]. Similar results have been obtained with other Notch targets in the nervous system, such as Hes-5, Hey-1 and Hey-2 [245-247]. These effects are most likely a consequence of persistent expression of pro-neuronal proteins such as Mash-1. Knock-out studies have shown that *Mash-1* is involved in promoting neuronal differentiation of already committed cells, as neuronal precursor cells still can be detected even though terminal differentiation into neurons is blocked[248].

Lateral inhibition and inductive signaling – a role for Notch in cell type specification

One process, by which Notch inhibits cells from adapting a default cell fate and maintaining a pool of multipotent progenitor cells is called lateral inhibition (Figure 11A) and was originally described in *D. Melanogaster*. In this model, Notch signaling occurs between adjacent cells in an initially homogenous progenitor pool expressing both Notch receptors and ligands. Undefined stochastic events will lead to increased ligand levels on one cell that will then activate Notch signaling in its neighbors. As Notch signaling is inhibitory for endogenous ligand expression**,** initially small differences in the receptor:ligand ratio will be amplified by a feedback mechanism and one cell will become signal sending (ligand expressing) and the other one signaling receiving (receptor expressing). In case of neural development, the signal sending cell will differentiate into a neuronal precursor cell whereas the signal receiving, and thus Notch expressing cell, will remain undifferentiated[212,235,249].

Another process by which Notch signaling inhibits a default cell fate is called inductive and restrictive cell fate determination. Apart from inhibiting neuronal differentiation and maintaining an undifferentiated progenitor pool, Notch activation in some contexts actually promotes a particular cell fate[205,250-252], thus instructing cells towards a specific cell fate (Figure 11B). This event occurs

between two developmentally distinct cells, one ligand and one receptor expressing cell, where ligand induced Notch activation instructs the latter to adopt a certain cell fate. It has as such been proposed that differentiation of certain types of glia such as radial glia and astrocytes are induced by Notch activation[251,253,254] during embryogenesis[255] or in the adult brain[256], respectively. In opposite, differentiation towards oligodendrocytes seems to be the default cell fate, as it is inhibited by Notch activation[251,252].

Taken together, activation of Notch can either act through lateral inhibition to inhibit a neuronal fate and maintain an undifferentiated progenitor pool or through instructive signaling to induce differentiation towards astrocytes while the lack of Notch activation results in oligodendrocytic differentiation. However, it is at present still not clear whether Notch acts sequentially on the same cells of the progenitor pool such as it first inhibits the neuronal fate and then instructs the remaining progenitor cells to become astrocytes or whether there are predetermined progenitors namely neuroblasts and glioblasts on which Notch acts[257]. Nevertheless, it is critical to note that timing is important in the outcome of Notch signaling, and as such it is only during certain phases of development that Notch activation supports the maintenance of undifferentiated progenitor cells at the expense of neurons, and generate astrocytes instead of oligodendrocytes[212,254,258].

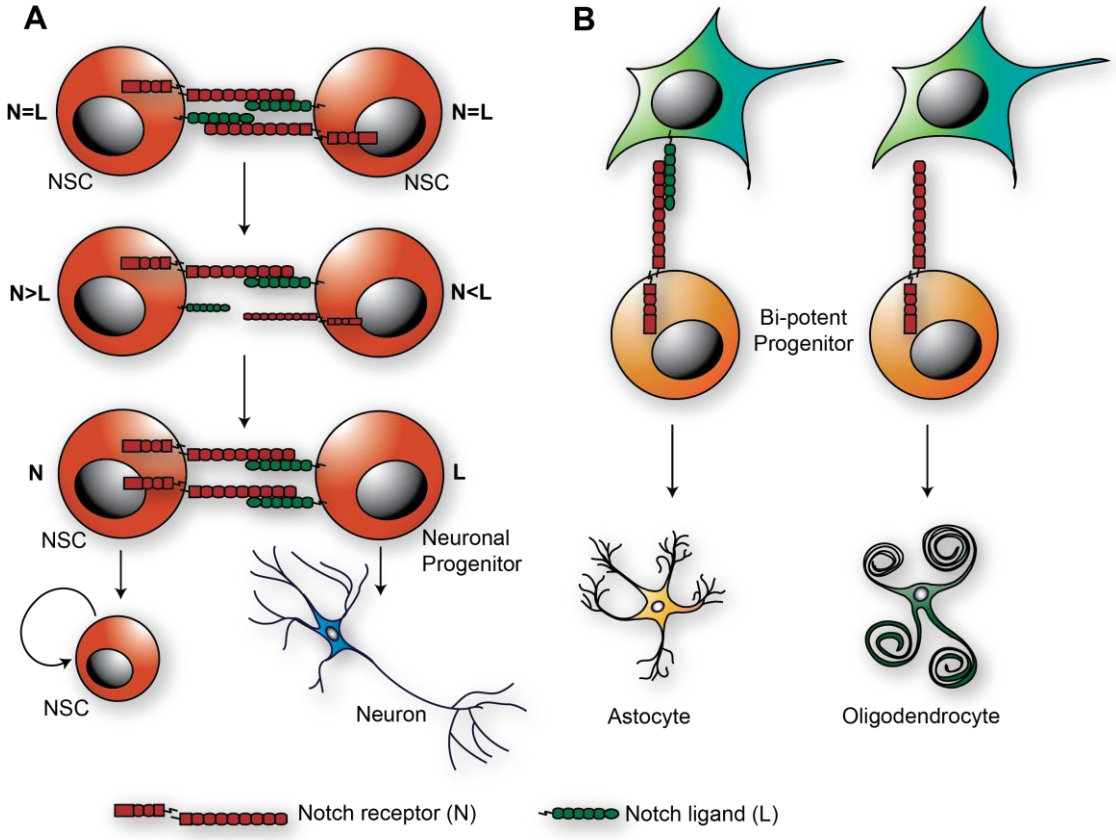

Figure 11: The role of Notch signaling in binary cell fate decisions. A) Lateral inhibition occurs between developmentally identical cells expressing equal amounts of both the Notch receptor (N) and the ligand (L). Stochastic events result in enhanced expression of either receptor or ligand in one of the cells, ultimately producing a Notch receptor expressing cell and a ligand expressing cell. The former will remain uncommitted and thus maintain the NSC pool, while the latter will be committed to the neuronal lineage. B) Inductive signaling occurs between two different cell types. A bi-potential, Notch expressing, progenitor cell is instructed to adopt a particular cell fate, e.g. astrocytic, upon interaction with a ligand expressing cell. In the absence of ligand induced Notch signaling, the bi-potential cell will adopt a default cell fate e.g. oligodendrocytic.

2.5.3 Notch in GBM and bCSC

Notch was first associated with tumorigenesis by the discovery of a constitutive activated mutated Notch receptor in acute T cell lymphoblastic leukemia (T-ALL)[259]. Although loss of function mutations of the Notch-2 receptor have been reported in a minor subset of GBM patients[260], mutations in the Notch receptors do not seem to be common event. Moreover, combined activation of KRAS and Notch using the RCAS/TVA model described in section 2.2.2 Holland *et al.* generated lesions along the SVZ, while activation of Notch alone failed to do so[230]. Nevertheless, components of the Notch pathway are often found aberrantly expressed in GBM and thereof derived *in vitro* cultures. Notch-1 and the corresponding intracellular Notch-1 domain (ICN-1) have generally been found up regulated in glioma cell lines and primary glioma samples as compared to normal non-neoplastic brain tissue[97,209,261,262]. Furthermore, the general expression levels of Notch-2 and -4, ligands and downstream target genes in glioma cell lines and primary glioma samples have been reported aberrant as compared to normal brain tissue[209,262,263]. However, the overall ligand expression has, not been found significantly elevated, most likely due to the highly variable ligand levels between different glioma grades [264]. Moreover, in the study by Lee *et al.* outlined in section 2.2.1, the cluster containing normal NSC and GBM neurosphere cultures intriguingly expressed high levels of genes involved in CNS function and development as well as stem cell associated genes such as Notch-1 and Dll-1 and -3[3]. By additional gene expression profiling, Günther *et al.* divided nine glioma cell lines established from GBM under serum-free conditions into two clusters: Cluster-1 was classified as having multipotent and sphere-forming potential, CD133 expression and high invasiveness, whereas cluster-2 had restricted differentiation potential, showed little or no CD133 expression and was less tumorigenic. The differently expressed transcripts were grouped with regard to their association to specific signaling pathways. Two of the transcripts over expressed in cluster-1 belonged to the Notch cascade whereas none of the cluster-2 cell lines showed increased expression of these genes[105]. In line with this, Mizutani *at al.* showed that a high *in vitro* Notch expression resulted in a higher frequency of sphere formation from normal NSC than when Notch expression was low[224]. The role of Notch signaling in bCSC maintenance is further supported in a study by Ignatova *et al.* By culturing cells from glioma grade III and IV tumors under NSC conditions they found a subset of cells able to form clonal spheres. During these serum-free culture conditions, the sphere cells were negative for the expression of Delta[g]. On the contrary when the cultures were exposed to serum and allowed to adhere, indicating differentiation, they gained Delta expression[11]. However, no change in Nestin expression was observed between the two culture conditions. As described above, Dll expression is seen in cells committed to the neuronal lineage[241], in line with the concept of lateral inhibition, and is in this case likely to be associated with a more differentiated phenotype. Taken together, there are several reports on expression of Notch pathway components in GBM cell lines, primary tumors and bCSC. And from the above outlined studies, it is tempting to speculate, that inhibition of Notch signaling leads to increased differentiation and decreased tumorigenecity by reducing the bCSC pool while an increased Notch activity is linked to a more undifferentiated phenotype and increased tumorigenecity.

[g] The authors use only this description

In the latter years, a number of studies have investigated the functional relevance of Notch signaling in gliomas and bCSC. Purow and co-workers showed for the first time that glioma cells were dependent on Notch-1, Dll-1 and Jagged-1 expression as knock-down of either the genes induced apoptosis and inhibited proliferation as well as prolonged the survival in an orthotopic mouse model[264]. In another study by Yin *et al.* glioma cell lines were stably transfected with delta-like ligand-1 (Dlk-1)[265], which is an atypical Notch ligand that shares homology with Dll-1, but lacks a critical receptor-binding domain[266]. Nevertheless, expression of Dlk-1 protein resulted in increased proliferation, loss of contact inhibition, enhanced anchorage-independent growth in soft agar and significantly greater capacity to migrate, together indicating increased aggressiveness. By using a Dlk-1 antibody they could block the Dlk-1 induced proliferation[265]. Similar results were obtained by Kanamori *et al.* who used an additional approach to modulate Notch signaling[209]. In this study they found that inhibition of Notch signaling in glioma cell lines, either by inhibiting the γ-secretase or exposing the cells to antisense Notch-1 or Notch-1 small interfering RNA (siRNA), resulted in suppressed growth, induced change in the morphology and induced expression of differentiation markers in cells exhibiting Notch pathway deregulation. However, no increase in apoptosis was detected therefore increased cell death could not account for the observed growth suppression[209]. By examining the level of the Notch-1 intracellular domain, Zhang *et al.* found the active receptor highly expressed in the SHG-44 glioma cell line, which also was the only cell line investigated that expressed Hes-5[261]. *In vitro* adherent growth of four glioma cell lines including SHG-44 revealed a higher proliferation rate for those cell lines that expressed ICN-1. By stably transfecting SHG-44 with ICN-1 they obtained a cell line, which grew significantly faster and had a significantly higher colony forming potential and generated more spheres when plated in serum-free media as compared to the parental and control cell lines transfected with an empty vector. The spheres formed in the assay were tested positive for Nestin, and could differentiate into the three neural lineages, indicating the presence of bCSC. This study was the first to examine the functional role of Notch signaling in glioma derived bCSC characteristics[261].

The above outlined studies were primarily performed on commercial glioma cell lines grown in the presence of serum, which according to Lee *et al.* is a rather poor model of human GBM. It has, however, been increasingly more common to establish and culture GBM cells during NSC conditions as described above. A commonly used approach for targeting Notch signaling, especially in pre-clinical studies is the use of γ-secretase inhibitors (GSI) that hinder the release of the intracellular Notch domain and thus transcription of target genes. In a recent study, Hu and colleagues showed that GSI treatment of both normal NSC- and patient derived glioma neurosphere cultures resulted in decreased formation of primary and secondary spheres as well as increased differentiation, possibly due to hampered proliferation and self-renewal of the sphere forming cells[267] together indicating Notch as an important player in maintaining the undifferentiated and tumorigenic potential of bCSC. By siRNA knock-down of the Notch-1 receptor in GBM cell lines grown as neurospheres Wang *et al.* were able to inhibit *in vitro* viability as well as *in vivo* subcutaneous tumor growth both when evaluating the tumor growth of cells treated prior to engraftment and when the siRNA was administered locally after the tumor had formed[268]. The effect of Notch inhibition on GBM cell viability is further supported in a study that targets the Notch downstream mediator Hes-3 using RNA interference (RNAi) resulting in reduced cell

number[269]. Taken together, these data suggest that the effect observed when inhibiting the Notch receptor, either by means of GSI or on a translational level, is most likely a consequence of abolishing signaling downstream from the receptor and thus the transcription of target genes. Fan and co-workers have previously shown that they could deplete CD133+ cells from an embryonal brain tumor medulloblastoma model by GSI treatment[270]. Using the same GSI (GSI-18) on GBM derived neurosphere cultures they obtained similar results as GSI treatment reduced cell viability and xenograft tumor growth both subcutaneously and orthotopic. In opposite, activating the pathway by transfection with the intracellular Notch-2 domain showed increased cell viability and tumor growth[26].

In conclusion, there are several lines of evidence indicating a functional role for Notch signaling in GBM, bCSC and glioma aggressiveness as studies indicate that an active Notch pathway is important for proliferation and maintenance of the undifferentiated tumorigenic phenotype of bCSC. In addition, Notch signaling has been implicated a role in bCSC radioresistance[271] and tumor angiogenisis[272,273] verifying the importance of Notch signaling in GBM tumorigenisis. Thus, Notch serves as a potential target for bCSC directed anti-GBM therapy as inhibition of Notch signaling has been demonstrated to abolish proliferation and induces differentiation in the bCSC and thereby eradicating this cell population.

3. Aim of the project

Objectives

GBM is today considered to be incurable as nearly all patients will experience relapse and die. Several indications appoint bCSC as the GBM cells responsible for tumor initiation, progression, treatment resistance and ultimately recurrence of the tumor. The EGFR and Notch signaling pathways are known to be important for maintaining the normal NSC population. These two pathways are often found aberrantly activated in GBM and recent reports suggest that they play a significant role in bCSC as well. However little is known about the specific function of the pathways in bCSC.

Hypothesis

By utilizing GBM neurosphere cultures established during stem cell culture conditions a representative *in vitro* GBM model for studying the functional role of EGFR and Notch activity in bCSC can be obtained.

Specific aims

1. Establish and characterize GBM neurosphere cultures based on their NSC-like characteristics and expression of EGFR and Notch pathway components
2. Establish an orthotopic GBM model by injecting neurosphere cells stereotactically into the brains of immunocompromised mice.
3. Investigate the functional relevance of EGFR and Notch activity in the neurosphere cells and identify possible predictive markers for response.

4. Results - Manuscript I

Maintenance of EGFR and EGFRvIII expression in an *in vivo* and *in vitro* model of human glioblastoma multiforme

Running title: EGFR and EGFRvIII expression in a GBM model

By:

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Research Article

Maintenance of EGFR and EGFRvIII expressions in an *in vivo* and *in vitro* model of human glioblastoma multiforme

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ABSTRACT

Glioblastoma multiforme (GBM) is the most common, and most aggressive primary brain tumor among adults. A vast majority of the tumors express high levels of the epidermal growth factor receptor (EGFR) as a consequence of gene amplification. Furthermore, gene amplification is often associated with mutation of EGFR, and the constitutive activated deletion variant EGFRvIII is the most common EGFR mutation found in GBM. Activated EGFR signaling, through overexpression and/or mutation, is involved in increased tumorigenic potential. As such, EGFR is an attractive target for GBM therapy. However, clinical studies with EGFR inhibitors have shown inconsistent results, and as such, further knowledge regarding the role of EGFR and EGFRvIII in GBM is needed. For this, an appropriate *in vivo*/*in vitro* tumor model is required. Here, we report the establishment of an experimental GBM model in which the expressions of EGFR and EGFRvIII are maintained both in xenograft tumors growing subcutaneously on mice and in cell cultures established in stem cell conditions. With this model it will be possible to further study the role of EGFR and EGFRvIII, and response to targeted therapy, in GBM.

Introduction

Glioblastoma multiforme (GBM) is the most aggressive primary brain tumor in adults with no current cure. The tumors belong to the group of astrocytic gliomas and appear either *de novo* without a previously diagnosed tumor, primary GBM, or arise from pre-existing low-grade tumors, secondary GBM [1]. Although clinically indistinguishable, primary and secondary GBM show some specific molecular features. Secondary GBM are frequently associated with mutation of the tumor suppressor gene p53, whereas primary GBM often show amplification and/or overexpression of the receptor tyrosine kinase epidermal growth factor receptor (EGFR) [2,3]. EGFR mutations are common in primary GBM and the deletion variant EGFRvIII is the most abundant. EGFRvIII is characterized by a deletion of 267 amino acids in its extracellular domain, including parts of the ligand binding region. This renders the receptor independent of ligand binding, and its resultant low constitutive activity has been correlated with increased malignant potential [4–6].

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The importance of EGFR and EGFRvIII expressions in tumorigenicity and GBM malignancy has been verified in several *in vivo* and *in vitro* studies. EGFR and EGFRvIII have been shown to increase malignancy and cell motility in animal models, and cells expressing EGFRvIII have also been shown to be more resistant to radiation therapy and EGFR inhibitors [7–13]. Thus, it is believed that EGFR and EGFRvIII might be involved in the genesis and/or progression of GBM, and EGFR is therefore considered to be an attractive target in GBM therapy. The two main approaches to target EGFR is through tyrosine kinase inhibitors (TKI) that bind to the intracellular domain of the receptor and inhibits its kinase activity, and humanized monoclonal mouse anti-EGFR antibodies that hinders ligand binding and receptor dimerization, which is crucial for its autophosphorylation and activity [14]. Although EGFR inhibition has been shown effective in several other cancer forms, studies regarding the prognostic and predictive role of EGFR and EGFRvIII in GBM have been inconclusive [15–22], and clinical studies with EGFR inhibitors in GBM have shown inconsistent results [23–25]. As such there is a need for further clarification of the role EGFR and EGFRvIII in GBM malignancy. One of the major concerns in evaluating the effect of EGFR inhibitors in GBM is the lack of an appropriate tumor model. GBM tumor cells grown *in vitro* invariably lose their *EGFR* amplification and overexpression as well as their EGFRvIII expression [26,27]. As such, the majority of studies on EGFR are performed with established GBM cell lines exogenously transfected with EGFR and EGFRvIII. Although these cells have revealed important information regarding the function of EGFR and EGFRvIII, they might not be suitable for studies investigating the response to targeted treatment, as these cells obviously are not dependent on EGFR amplification/overexpression or EGFRvIII expression for their survival.

During the last couple of years the concept of brain cancer stem-cells (bCSC) has gained much attention in GBM malignancy. These cells show profound similarity to normal neural stem cells (NSC) as they express NSC markers and are able to differentiate into the three neural lineages, namely astrocytes, oligodendrocytes and neurons [28–32]. When grown in a defined stem cell media, the bCSC form neurospheres, as a result of their self-renewing capacity. Several studies have shown that the bCSC are responsible for tumor growth and relapse, and as such are an interesting target in GBM therapy [28,30,31]. Furthermore, in a recent study by Lee et al., it was shown that GBM cell cultures established in stem cell media were more representative for the original patient tumor than cell lines established in the presence of serum [33]. Thus it is feasible that cultures established in stem cell conditions enrich for the bCSC population, and as this population of cells has been implicated in the tumorigenesis of GBM, these cells would also maintain important characteristics of GBM tumors that are otherwise lost in traditionally grown cell cultures.

In this study we report the establishment of a representative GBM model retaining the EGFR and EGFRvIII expressions of the original patient tumor even after several *in vivo* and *in vitro* passages. Tumor material obtained at routine operation was subcutaneously transplanted into the flanks of nude mice. Once established as serially transplantable xenografts, tumors were analyzed for EGFR and EGFRvIII expressions and found to maintain the expression even after several passages. These xenografts were subsequently used to establish cell cultures *in vitro*. We show that cell cultures established in stem cell conditions maintain the EGFR and EGFRvIII expressions even after several months in culture. These cells, in combination with the xenograft tumors can now be used as a representative *in vivo* and *in vitro* model for GBM to further elucidate the role of EGFR and EGFRvIII and to study the response to targeted EGFR therapy.

Materials and methods

Patients and in vivo growth

Tumor material was obtained during surgery at Copenhagen (CPH) University Hospital, Denmark and was approved by the Scientific Ethical Committee for Copenhagen and Frederiksberg (KF 01-034/04). Tumors were diagnosed as GBM according to the WHO 2000/2007 guidelines. Tumor xenografts were generated by subcutaneous transplantation of approximately 1 mm^3 pieces of tumor tissue into the flanks of 6-week-old female NMRI nude mice (Taconic, Ry, Denmark). This was called xenograft passage 1 (p1). When reaching maximal size, the tumors were passaged by cutting the tumor into 1 mm^3 pieces and transplanted subcutaneously (p2, p3, etc.…). Simultaneously, tumor tissue was collected for further analyses and verification of tumor tissue. For *in vivo* evaluation of tumorigenicity of *in vitro* cultured cells, 2×10^6 cells were subcutaneously inoculated into the flanks of nude mice (NMRI nude mice, Taconic, Ry, Denmark). At the end of experiment, tumor tissue was collected for further analyses. For assessment of tumor growth, tumors were measured in two perpendicular dimensions (d1 and d2) and tumor area, $A = d1 * d2$ was calculated. Tumor volume was then calculated using the formula: $V = \Pi/6 * A3/2 * k$, where k is a constant that describes the relationship between d1 and d2 and the third dimension ("height") of the tumor. Tumor growth curves were established using the Gompertz function as has been described earlier [34].

Tumor xenografts were passaged over nude rats (RNU Nude Rat, Charles River Laboratories International, Inc., Wilmington, MA) in order to exterminate mouse hepatitis infection. All xenografts were tested with the Mouse RapidMAP 27 test (Taconic, Ry, Denmark), before they were again maintained as xenografts on the flanks of nude mice. Hereafter the tumors were assigned the prefix "N". Tumor xenografts free of mouse hepatitis infection were: GBM_CPH014, GBM_CPH017, GBM_CPH029, GBM_CPH036, GBM_CPH047 and GBM_CPH048.

Cell culture

In vitro cultures of GBM cells were established from xenograft GBM tumors free of mouse hepatitis infection. Xenograft GBM tumors were mechanically cut into small pieces (explants) that were covered with one drop of Neurobasal media (NB) (Invitrogen, Taastrup, Denmark) with additives: N2, B27, bFGF (10 ng/ml), EGF (10 ng/ml), L-glutamine, penicillin (50 U/ml) and streptomycin (50 µg/ml) (all from Invitrogen, Taastrup, Denmark) per piece and allowed to adhere to the tissue culture dish surface. For NGBM_CPH036p7 and NGBM_CPH048p6, enzymatic dissociation using 1× Accutase (Millipore, Copenhagen, Denmark) into single cells was additionally performed prior to plating and LIF (10 ng/ml) (Millipore, Copenhagen, Denmark) was used instead of N2. When migrating cells were detected, both adherent cells and cells in suspension were collected and called passage 1. Fresh media was added twice a week and spheres were mechanically dissociated at

every passage. The U87MG cell line was obtained from the American Type Culture Collection. U87MGvIII and U87MG-EGFR cell lines were kindly provided by Dr. Webster Cavanee (Ludwig Institute for Cancer Research, San Diego, CA). The NR6M and NR6W cell lines were a gift from Dr. Darrell Bigner (Duke University Medical Center, Durham, NC) and the NR6WA cell line was a gift from Dr. Alan Wells (University of Pittsburgh, Pittsburgh, PA). The U87MG, U87MGvIII, U87MG-EGFR, NR6M, NR6W and NR6WA cell lines were used as positive controls for expressions of EGFR and EGFRvIII.

Western blotting

Whole cell protein lysates (5 μg) were separated on 3–8% NuPAGE TA gels (Invitrogen, Taastrup, Denmark) and electroblotted onto nitrocellulose membranes (Invitrogen, Taastrup, Denmark). The membranes were then blocked for 1 h at room temperature (RT) and incubated with primary antibodies in 5% non-fat milk overnight (ON) at 4 °C followed by secondary antibodies for 1 h at RT. Blots were developed using the SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL) and the Biospectrum Imaging System (UVP, Upland, CA). Primary antibodies used: goat polyclonal anti-EGFR (20-ES04, Fitzgerald Industries International, Concorde, MA), mouse monoclonal anti-EGFRvIII (clone DH8.3, Novocastra, Newcastle upon Tyne, UK), mouse monoclonal anti-EGFRvIII (L8A4, a kind gift from Dr. Darrell Bigner, Duke University Medical Center, Durham, NC) and rabbit anti-GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). EGFR expression was graded on a scale from 0 to 3 where neg = no expression; x = low expression; xx = medium expression and xxx = high expression, as judged from band intensity.

Polymerase chain reaction (PCR)

Total RNA was purified with the RNeasy Mini kit and QIAshredder (both from Qiagen Denmark, Copenhagen, Denmark) as described by the manufacturer. RNA from tumor tissue samples was extracted with TRIzol®Reagent (Invitrogen, Taastrup, Denmark) prior to purification with the RNeasy kit. All RNA was DNase treated using the RNase-Free DNase Set from Qiagen Denmark (Copenhagen, Denmark) according to the manufacturer's instructions. cDNA was synthesized using the SuperScript™ III Platinum® Two step qRT-PCR kit with SYBR® Green (Invitrogen, Taastrup, Denmark) which was also used for quantitative real-time PCR (Q-RT-PCR) reactions. For non-quantitative PCR reactions Platinum Taq Polymerase (5 U/μl) (Invitrogen, Taastrup, Denmark) was used. Relative quantification of Q-RT-PCR expression levels was performed according to the comparative Ct method including the efficiency of the primers. All data was normalized to the expression of three housekeeping genes (*TOP1*, *EIF4A2* and *CYC1*) included in the human geNorm housekeeping gene selection kit (Primerdesign, Southampton, UK). PCR reactions were optimized to distinguish between *EGFR* and *EGFRvIII* and to be specific for human genes, if not stated otherwise. Genes analyzed were: *EGFR* forward, 5′-TCC TTG GGA ATT TGG AAA TT-3′; *EGFR* reverse, 5′-GGC ATA GGA ATT TTC GTA GTA CAT-3′; *EGFRvIII* forward, 5′-ATG CGA CCC TCC GGG ACG-3′; *EGFRvIII* reverse, 5′-ATC TGT CAC CAC ATA ATT ACC T-3′ [16]; *GFAP* forward, 5′-CGC TGG TAG AGA TGG AGG AG-3′; *GFAP* reverse, 5′-CTG GGG TTA AGA AGC AGC AG-3′; mouse *ACTIN* forward, 5′-TTT GTT TTG TTTT GGC GCT T-3′; mouse *ACTIN* reverse; 5′-GGG CCA TTC AGA AAT TAA AA-3′, *ACTIN* forward, 5′-GTC GAC AAC GGC TCC GGC ATG TGC A-3′; *ACTIN* reverse, 5′-GCC AGC CAG GTC CAG ACG CAG GAT G-3′; *CD31* forward, 5′-CCC GAA GGC AGA ACT AAC TG-3′; and *CD31* reverse, 5′-GGG TCA GGT TCT TCC CAT TT-3′.

Immunohistochemistry (IHC) and immunocytochemistry (ICC)

Patient tumor tissue and tumor xenografts were formalin-fixed and paraffin-embedded. All tumors were stained with haematoxylin and eosin (HE) to verify tumor diagnosis. For immunocytochemistry (ICC), cells were washed in PBS and centrifuged at 350 ×*g* for 5 min. The resultant pellet was formalin-fixed and paraffin-embedded. Formalin-fixed and paraffin-embedded slides (4 μm) were melted for 1 h at 60 °C, followed by de-paraffination in xylene and ethanol. Endogenous peroxidase was blocked in hydrogen peroxide 3% for 10 min. Afterwards the slides were de-masked in MBO-TEG for 15 min (except for TissuGnost® EGFR (E30) for which Proteinase K

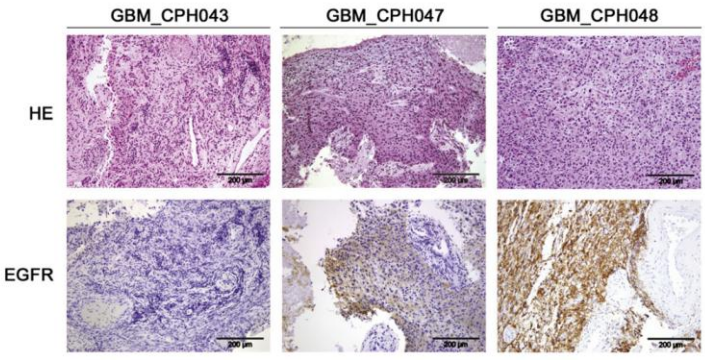


Fig. 1 – EGFR expression in GBM tumors. Representative EGFR IHC stainings of patient GBM tumors included in the study. EGFR is visualized as a brown staining, whereas nuclei are stained blue. GBM_CPH043 = neg; GBM_CPH047 = xx and GBM_CPH048 = xxx. HE staining was used to confirm diagnosis and presence of tumor tissue. ×200 magnification.

was used for 5 min). Incubation with the following primary antibodies: TissuGnost® monoclonal mouse anti-EGFR (E 30) (1:200 dilution, Merck KGaA, Darmstadt, Germany), rabbit monoclonal anti-EGFR (15F8) (1:50 dilution, Cell Signaling Technology, Inc., Danvers, MA) (used for detection of EGFR in tumor xenografts), polyclonal rabbit anti-GFAP (1:6400 dilution), monoclonal mouse anti-Vimentin (1:1600 dilution), and monoclonal mouse anti-Neurofilament (NF) (1:4000 dilution), all from DakoCytomation, Glostrup, Denmark, monoclonal mouse anti-Nestin (1:800 dilution), and monoclonal mouse anti-NeuN (1:200 dilution) from Millipore, Copenhagen, Denmark and monoclonal mouse anti-CD56 (NCAM) (1:200 dilution, Novocastra, TriChem Aps-interkemi, Skanderborg, Denmark) was performed for 30 min at RT, for EGFR 15F8, blocking in 2% BSA was performed prior to addition of the primary antibody. Subsequently, EnVision + System-HRP Labelled Polymer (anti-mouse or anti-rabbit) was applied for 30 min followed by DAB+-chromogen for 10 min, both from DakoCytomation, Glostrup, Denmark. Enhancement was performed with 0.5% $CuSO_4$. All sections were counterstained with Mayer's Haematoxylin and were performed on a DAKO Cytomation Autostainer Plus (DakoCytomation, Glostrup, Denmark). Evaluation of the slides was performed independently and under blind conditions by H.B. (Neuropathologist, MD) and M.T.S (Senior Scientist, PhD). Staining of cells was graded as follow: neg = 0%; x = 1%–10%; xx = 11%–50%, xxx = >50% of the cells stained positive.

Fluorescence in situ hybridization (FISH)

EGFR fluorescence in situ hybridization (FISH) on paraffin sections from patient tumors and xenografts was performed by Medical Solutions, UK. In brief, samples were hydrolyzed in HCL acid for 29 min before being placed in pre-treatment solution for 45 min at 87 °C, and digested with protease solution for 30 min at 37 °C. Slides were allowed to dry where after the EGFR/Chromosome 7 (CEP7) probe was added and allowed to hybridize for at least 14 h. Posthybridization wash was performed in a water bath at 69 °C where after the slides were allowed to dry at 70 °C for 5 min. Tissue sections were covered with 4′6-diamidino-2-phenylindole (DAPI) for chromatin counterstaining before microscopy. Analyses were done with a fluorescence microscope (Leica DMLB) (manual scoring—no image analysis). EGFR was visualized as a red signal with a Spectrum Orange filter, CEP7 as a green signal with a Spectrum

Table 1 – EGFR and EGFRvIII expressions in patient material.

Patient	Diagnosis	EGFR IHC	EGFR WB	EGFRvIII WB	Xenograft	*In vitro* growth
GBM_CPH001	GBM	xxx	xx	neg	−	−
GBM_CPH002	GBM	neg	x	neg	−	−
GBM_CPH003	GBM	neg	x	neg	−	−
GBM_CPH004	GBM	neg	xx	neg	−	−
GBM_CPH006	GBM	xxx	xxx	pos	−	−
GBM_CPH009	GBM	neg	x	neg	−	−
GBM_CPH011	GBM	xx	x	neg	−	−
GBM_CPH014	GBM	neg	x	neg	+	−
GBM_CPH017	GBM	x	x	neg	+	+
GBM_CPH018	GBM	xx	x	neg	−	−
GBM_CPH019	GBM	xx	xx	neg	+	n.a
GBM_CPH020	GBM	neg	neg	neg	−	−
GBM_CPH021	GBM	xx	x	neg	+	n.a
GBM_CPH022	GBM	x	xx	neg	−	−
GBM_CPH023	GBM	neg	x	neg	+	n.a
GBM_CPH024	GBM	xxx	xxx	pos	+	n.a
GBM_CPH025	GBM	x	neg	neg	+	n.a
GBM_CPH028	GBM	xxx	xxx	pos	+	n.a
GBM_CPH029	GBM	xx	x	pos	+	+
GBM_CPH031	GBM	neg	neg	neg	−	−
GBM_CPH033	GBM	xxx	xxx	neg	+	n.a
GBM_CPH035	GBM	xxx	xxx	neg	+	n.a
GBM_CPH036	GBM	neg	x	neg	+	+
GBM_CPH037	GBM	neg	x	neg	−	−
GBM_CPH038	GBM	xxx	xx	neg	−	−
GBM_CPH040	GBM	xxx	xx	neg	+	n.a
GBM_CPH042	GBM	neg	x	neg	−	−
GBM_CPH043	GBM	neg	neg	neg	−	−
GBM_CPH045	GBM	neg	xxx	neg	−	−
GBM_CPH046	GBM	xxx	xxx	neg	+	n.a
GBM_CPH047	GBM	xx	neg	neg	+	+
GBM_CPH048	GBM	xxx	xxx	neg	+	+
GBM_CPH049	GBM	neg	neg	neg	−	−
GBM_CPH051	GBM	neg	x	neg	−	−
GBM_CPH052	GBM	xx	xx	neg	+	n.a
GBM_CPH053	GBM	x	xx	neg	+	n.a

n.a. = not attempted.

Green filter and nuclei as a blue signal with a DAPI filter. Up to 200 nuclei in five different fields were enumerated, ×100 Magnification.

Multiplex ligation-dependent probe amplification (MLPA)

DNA was extracted from patient tumors, xenografts and cell cultures using TRIzol®Reagent (Invitrogen, Taastrup, Denmark) according to the manufacturer's instructions. In brief, Trizol was added to the samples and homogenized with Qiagen Tissue Lyser (Qiagen Denmark, Copenhagen, Denmark). After lysis, chloroform was added and DNA isolation continued from the inter- and phenol phases. DNA was precipitated using 100% ethanol, centrifuged and the resultant pellet was washed in 0.1 M Sodium citrate in 10% ethanol. After a final wash in 75% ethanol, the pellet was air-dried and resuspended in TE buffer. DNA was extracted from peripheral blood from healthy donors using the same procedure with the exception for an additional step including removal of red blood cells from the samples by lyzing with RIPA-buffer followed by centrifugation, before addition of the TRIzol®Reagent. Multiplex Ligation-Dependent Probe Amplification (MLPA) reactions were performed on genomic DNA using the commercial SALSA MLPA kit P105-C1 Oligodendroglioma-2 according to the manufacturer's instructions (MRC-Holland, Amsterdam, The Netherlands). PCR products were analyzed on a 3130x*l* Genetic Analyzer (Applied Biosystems, Foster City, CA). The resulting electropherogram data were quantified by GeneMapper Software 4.0 (Applied Biosystems, Foster City, CA), and the final quantified MLPA data were analyzed by an in-house developed software [35] (details and downloads of the software are available on www.chromosomelab.dk). DNA from 9 normal controls was included for normalization of the samples and DNA extracted from mouse muscle was initially tested to ensure specificity for human DNA.

Results

EGFR and EGFRvIII expressions in patient material

Tumor material obtained at routine surgery was subcutaneously xenografted into the flanks of nude mice. In parallel, the tumor tissue was verified as GBM by histological evaluation and stained for the expression of EGFR by immunohistochemistry (IHC). HE and EGFR stainings of representative GBM tumors are shown in Fig. 1. GBM_043 was scored to be negative for EGFR expression whereas GBM_CPH047 and GBM_CPH048 were scored as positive. In high expressing tumors such as GBM_CPH048, the expression of EGFR was uniform and could be detected in almost all tumor cells whereas infiltrative vessels were negative. In contrast, EGFR distribution in tumors that expressed medium levels of EGFR, such as GBM_CPH047, was heterogeneous. Expressions of EGFR and EGFRvIII were also analyzed by Western blotting (WB), and the results of the IHC stainings and WB analyses are presented in Table 1. In most cases the IHC and WB results are well corresponding; however in some cases they do not correlate. This is most probably due to the observed heterogeneity of EGFR expression as mentioned above.

GBM xenograft tumors maintain the expressions of EGFR and EGFRvIII

Out of 36 evaluable patients in the study, 18 tumors were established as xenografts on mice resulting in a 50% take-rate (Table 1). The xenografts were maintained by serial passaging and the growth of each tumor was monitored by measuring its size in two perpendicular dimensions, and tumor area was calculated.

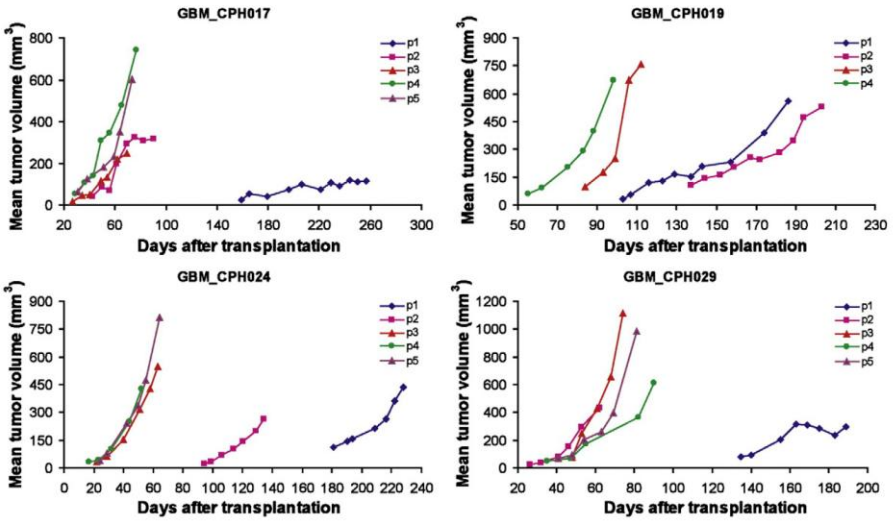


Fig. 2 – Growth curves of representative tumor xenografts. Tumor size was measured in two perpendicular dimensions and tumor volume was calculated as described in the Materials and methods section. Tumor growth curves were established using the Gompertz function. p1 = xenograft passage 1, p2 = xenograft passage 2, etc....

Growth curves were established as described in the materials and methods section. Fig. 2 shows representative growth curves from four different xenografts. In most cases the lag-period before visible tumors were detected decreased, and the tumor growth rate increased, with increasing passages. In most cases the tumor growth was stabilized after repeated serial passaging, however xenografts derived from different patient tumors continued to show substantial variation in growth rate. This is consistent with what has been reported by others [27].

At the time of passaging, xenograft tumor tissue was collected for analysis of EGFR and EGFRvIII expressions by WB, IHC and Q-RT-PCR, and the tumors were confirmed to be GBM by histological analysis. As shown in Fig. 3A, EGFR and EGFRvIII protein expressions were maintained over several *in vivo* passages. In GBM_CPH048 a rearrangement of EGFR could be detected as shown by increased protein size. IHC stainings of EGFR were very well corresponding to the WB results and are presented in Table 2. We furthermore analyzed *EGFR* and *EGFRvIII* mRNA expressions (Figs. 3B and C). For this purpose EGFR and EGFRvIII specific primers were developed, ensuring that a) only human transcripts were detected and b) that the reactions were specific for either *EGFR* or *EGFRvIII* transcripts [16]. The results confirmed the results obtained from the WB and IHC analyses, showing that *EGFR* and *EGFRvIII* expressions were maintained even after several passages *in vivo* (Figs. 3B and C). In some cases, *EGFR* and *EGFRvIII* expressions were lower in the patient material than in the xenografts. This is most likely due to that the tumor tissue obtained from the patients contain a mixed cell population composed of both tumor cells and surrounding stroma, whereas the xenografts are solid tumors, with little or no infiltration of normal tissue. However, it could also reflect differences in the handling time between the xenograft tissue and the patient material.

High expression of EGFR and response to EGFR inhibitors has been associated to amplification of the *EGFR* gene [21,36–40]. As such it is of importance that the xenograft tumors maintain the *EGFR* amplification, if any, present in the patient tumor. Therefore we investigated the amplification status of *EGFR* in the diagnostic patient material and derived GBM xenografts by FISH analysis. Fig. 4 shows a representative picture of *EGFR* amplification in GBM_CPH017 patient material. In all cases analyzed, the *EGFR* amplification observed in the patient tumor was maintained in the xenografts (Table 3).

Cells from GBM xenografts grow as spheroids in stem cell media

As mentioned above, GBM cell cultures established in a well-defined stem cell media are more representative for the original patient tumor than cell cultures established in traditional serum containing conditions [33]. Therefore, once established as growing

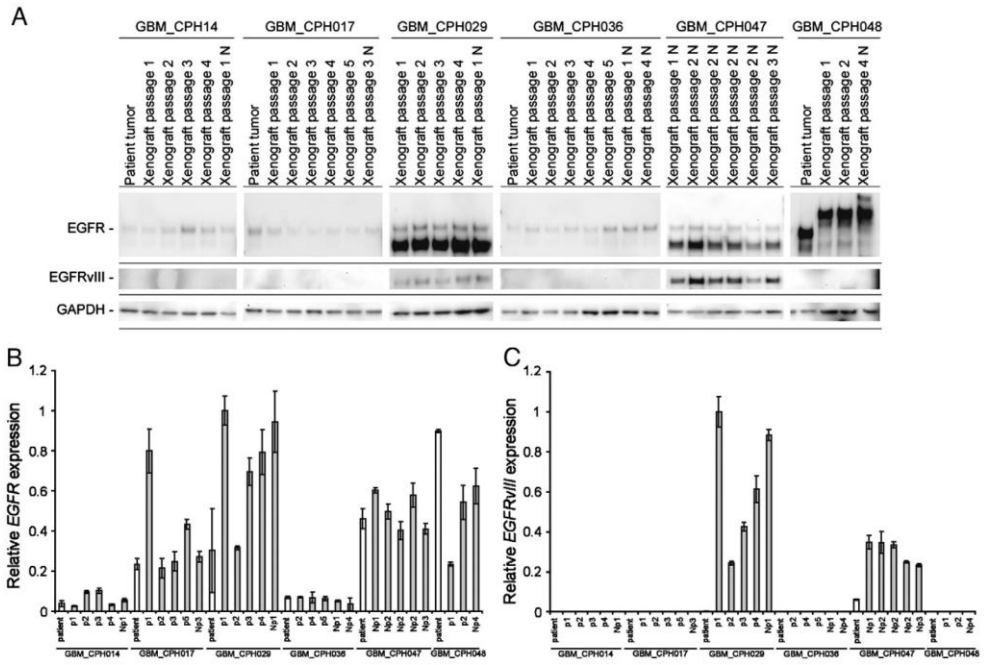


Fig. 3 – EGFR and EGFRvIII expressions are maintained *in vivo*. EGFR and EGFRvIII expressions in patient material and derived xenografts from several passages as assessed by A) Western blotting and B) and C) Quantitative Real-Time PCR (Q-RT-PCR). White bars = patient tumor, gray bars = xenografts. Q-RT-PCR reactions are presented as mean ± SD. "N" = xenograft tumors that have been passaged over nude rats. For detection of EGFRvIII by Western blotting in GBM_CPH014, GBM_CPH017, GBM_CPH029, GBM_CPH036 and GBM_CPH048 tumors, the mouse monoclonal anti-EGFRvIII clone DH8.3 from Novocastra was used and for GBM_CPH047 tumors, the mouse monoclonal anti-EGFRvIII L8A4 antibody was used.

Table 2 – IHC of EGFR in GBM tumors and xenografts.

Patient	Passage	EGFR
GBM_CPH014	Patient	neg
	P1	neg
	P4	x
	P1 N	neg
GBM_CPH017	Patient	x
	P1	neg
	P5	neg
	P3 N	neg
GBM_CPH029	Patient	xx
	P1	xx
	P3	xx
	P1 N	xx
GBM_CPH036	Patient	neg
	P1	x
	P3	x
	P4 N	x
GBM_CPH048	Patient	xxx
	P1	xxx
	P2	xxx
	P4 N	xxx

Table 3 – Summary of EGFR FISH analysis.

GBM_CPH014 patient	Low polysomy
NGBM_CPH014 xenograft p1	Low trisomy
GBM_CPH017 patient	High polysomy–gene amplification
NGBM_CPH017 xenograft p3	Low polysomy–gene amplification
GBM_CPH029 patient	Low polysomy–gene amplification
NGBM_CPH029 xenograft p1	High polysomy–gene amplification
GBM_CPH036 patient	Low trisomy
NGBM_CPH036 xenograft p4	Low trisomy
GBM_CPH048 patient	High polysomy–gene amplification
NGBM_CPH048 xenograft p4	High polysomy–gene amplification

Disomy–(≤2 copies >90% of cells).
Low trisomy–(≤2 copies ≥40% of cells; 3 copies in 10–40%; ≥4 copies in <10%).
High trisomy–(≤2 copies ≥40% of cells; 3 copies in >40%; ≥4 copies in <10%).
Low polysomy–(≥4 copies in 10–40% of cells).
High polysomy–(≥4 copies in ≥40% of cells).
Gene amplification (presence of tight EGFR gene clusters and gene to chromosome ratio of ≥2 or ≥15 copies per cell in ≥10% of cells).

xenografts, tumor explants were seeded in supplemented neurobasal media (NB) in order to allow cells to grow *in vitro*. Cells were found to grow either as spheres in suspension or as mixed cultures with spheres and adherent cells, which has also been observed by others [41] (Fig. 5A and data not shown).

One could argue that also infiltrating mouse cells, present in the xenograft tumors, would grow *in vitro*. Therefore primers specific for mouse *ACTIN* were developed and all cultures were analyzed with RT-PCR. As seen in Fig. 5B, the positive control, mouse muscle, was clearly positive for mouse *ACTIN*, whereas the negative controls, HMVEC (human microvascular endothelial cells) and CCD32lu (non-transformed human lung fibroblasts) were negative. A slight positive result was seen in human brain, however this was probably due to contamination of the sample. Importantly, all GBM cell cultures established in NB were negative for mouse *ACTIN*, showing that these cells are indeed of human origin. Furthermore, all cell cultures were negative for *CD31*, optimized to be specific for human endothelial cells. Thus the cell cultures established are most likely not derived from neither mouse nor human endothelial cell origin. In addition, all cell cultures investigated were positive for *glial fibrillary acidic protein* (*GFAP*), an intermediate filament of astrocytes that is highly expressed in astrocytic tumors such as GBM but that also is expressed by neural stem cells [42] (Table 4 and data not shown).

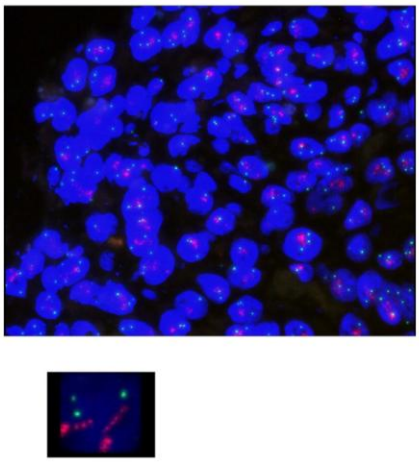


Fig. 4 – EGFR is amplified in GBM tumors. Representative pictures of GBM_CPH017 patient material showing amplification of the *EGFR* gene as detected by FISH analysis.

To further characterize the cells as GBM derived, we performed immunocytochemical (ICC) stainings of markers known to be expressed by gliomas and cells of the neuronal lineages. As summarized in Table 4, all cell cultures were positive for Vimentin, CD56 and Nestin, and all except for NGBM_CPH048p6 NB were positive for NeuN, whereas they were all negative for Neurofilament (NF). In addition to the RT-PCR analysis of *GFAP* described above, we also stained the cells for the expression of GFAP. As can be seen in Table 4, all cultures except for NGBM_CPH017p4 NB were positive for GFAP. Representative stainings of the different markers are shown in Fig. 5C. Expressions of Vimentin and Nestin were detected in almost all cells whereas GFAP, NeuN and CD56 expressions were more heterogeneous. Taken together these results show that the cell cultures established indeed are of GBM origin.

Cell cultures established in stem cell media maintain the expressions of EGFR and EGFRvIII

One major concern of GBM cell lines is that they lose their expressions of EGFR and EGFRvIII present *in vivo* when grown *in vitro* [26,27]. Therefore we tested the expressions of *EGFR* and *EGFRvIII* by Q-RT-PCR in our established cell cultures and the corresponding xenograft tumors from which they were derived. As can be seen in Fig. 6A all cell cultures investigated expressed *EGFR* at the same level as the matched xenografts, except for NGBM_CPH048p5 in which *EGFR* expression was much higher in the cell culture than in the parental xenograft. Furthermore,

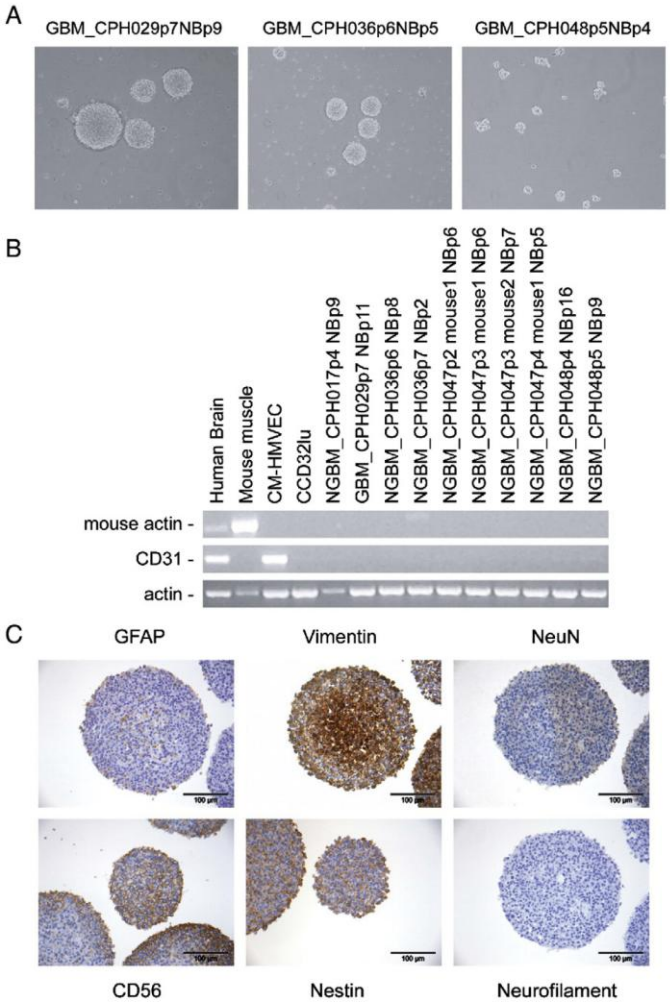


Fig. 5 – Cells cultured in stem cell conditions grow as neurospheres and are of human and astrocytic/neuronal origin. A) Representative pictures of neurospheres from GBM_CPH029p7NB, NGBM_CPH036p6NB and NGBM_CPH048p5NB. ×100 magnification. B) RT-PCR of established cell cultures showing that none of the cell cultures are positive for mouse *ACTIN* or *CD31*. C) Representative ICC stainings of markers of the neuronal and astrocytic lineages on neurospheres derived from NGBM_CPH036p6NBp2 showing spread positivity for GFAP, NeuN, and CD56, whereas staining for Vimentin and Nestin was detected in almost all cells. Staining for Neurofilament was negative. ×400 magnification. NB = cells grown in Neurobasal media as described in the Materials and methods section.

EGFRvIII expression was maintained in cell cultures established from *EGFRvIII* positive xenograft tumors (Fig. 6B). By WB we could also show that NGBM_CPH048 xenografts expressed a rearranged EGFR protein of two different sizes, as is shown in Fig. 6C. Cell cultures established from these xenografts seemed to favor the expression of one of the rearranged proteins (Fig. 6C). In addition, we also investigated the protein expression of EGFRvIII in NGBM_CPH047 xenografts and derived cell cultures. As shown in Fig. 6D, all xenografts and cell cultures investigated expressed EGFRvIII at comparable levels. By MLPA it is possible to screen for deletions and gains of specific genes. To investigate whether amplification of *EGFR* was maintained *in vitro* we thus analyzed our GBM cell cultures and their corresponding xenograft tumors with MLPA. From the results presented in Table 5 it is clear that cell cultures established from xenograft tumors with *EGFR* amplification also had gain of *EGFR in vitro* as exemplified by NGBM_CPH048p4 and p5. Even though in most cases the EGFR ratio was lower *in vitro* than *in vivo*. These results are also in good

Table 4 – Characterization of established cell cultures.

Cell culture	Mouse actin (PCR)	CD31 (PCR)	CD133 (PCR)	GFAP (PCR/IHC)	Vimentin (IHC)	NeuN (IHC)	CD56 (IHC)	Nestin (IHC)	NF (IHC)
NGBM_CPH017p4 NBp9	neg	neg	pos	pos/neg	pos	pos	pos	pos	neg
GBM_CPH029p7 NBp10, p11	neg	neg	pos	pos	pos	pos	pos	pos	neg
NGBM_CPH036p6 NBp2, p8	neg	neg	pos	pos	pos	pos	pos	pos	neg
NGBM_CPH036p7 NBp2, p12	neg	neg	pos	pos	pos	neg	pos	pos	neg
NGBM_CPH047p2mousel NBp6	neg	neg	pos	pos	pos	pos	pos	pos	neg
NGBM_CPH047p3mousel NBp6	neg	neg	pos	pos	pos	pos	pos	pos	neg
NGBM_CPH047p3mouse2 NBp7	neg	neg	pos	pos	pos	(pos)	pos	pos	neg
NGBM_CPH047p4mousel NBp5	neg	neg	pos	pos	pos	pos	pos	pos	neg
NGBM_CPH048p4 NBp16	neg	neg	pos	pos	pos	pos	pos	pos	neg
NGBM_CPH048p5 NBp9	neg	neg	pos	pos	pos	(pos)	pos	pos	neg
NGBM_CPH048p6 NBp12	neg	neg	pos	pos	pos	neg	pos	pos	neg

correlation with the data obtained from the FISH analysis described above (Table 3).

Cells grown in stem cell media are tumorigenic and maintain EGFR expression

In order to test whether the cell cultures established in stem cell media were tumorigenic we subcutaneously injected cells from the GBM_CPH029p7 NB, NGBM_CPH036p6 NB and NGBM_CPH047p3m1 NB cell cultures into the flanks of nude mice. At the end of experiment, tumors had developed in all mice. EGFR and EGFRvIII protein and mRNA expressions were analyzed by WB and Q-RT-PCR. As can be seen in Fig. 7A, EGFRvIII expression was not detected in the original GBM_CPH029 xenograft tumor at passage 7, and as such neither in the established cell culture nor in the resulting xenograft tumor. On the other hand, EGFR could be readily detected both in the NB

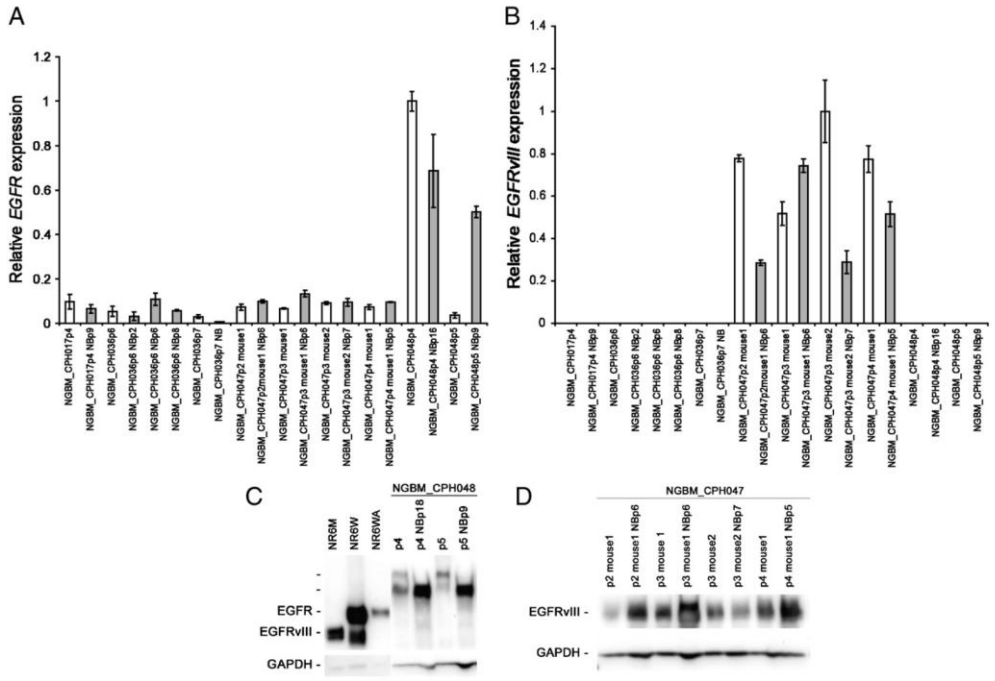


Fig. 6 – EGFR and EGFRvIII expressions are maintained *in vitro*. Quantitative Real-Time PCR analyses of A) EGFR and B) EGFRvIII in tumor xenografts (white bars) and derived cell cultures (gray bars). Q-RT-PCR reactions are presented as mean ± SD. C) EGFR expression in NGBM_CPH048 xenografts and cell cultures. D) Western blot analysis of EGFRvIII expression in cell cultures derived from NGBM_CPH047 xenografts of different passages. For detection of EGFRvIII by Western blotting, the mouse monoclonal anti-EGFRvIII L8A4 antibody was used. NB = Neurobasal media. NR6 are mouse fibroblasts expressing EGFRvIII (M), high levels of EGFR (W) and medium levels of EGFR (WA).

Table 5 – EGFR ratio in xenografts and derived cell cultures.

	EGFR ratio	P-value *
NGBM_CPH0I7p4	2.08	0.0033
NGBM_CPH0I7p4 NBp8	1.89	0.0000
NGBM_CPH036p6	1.43	0.0050
NGBM_CPH036p6 NBp6	1.61	0.0002
NGBM_CPH047p2 mousel	7.32	0.0104
NGBM_CPH047p2 mousel NBp8	2.94	0.0030
NGBM_CPH047p3 mousel	7.03	0.0112
NGBM_CPH047p3 mousel NBp6	4.55	0.0080
NGBM_CPH047p4 mousel	10.35	0.0108
NGBM_CPH047p4 mousel NBp5	4.46	0.0065
NGBM_CPH048p4	23.53	0.0001
NGBM_CPH048p4 NBp16	11.41	0.0000
NGBM_CPH048p5	25.96	0.0000
NGBM_CPH048p5 NBp11	9.65	0.0000

* One-tailed P-values. Mean ratios were tested for being outside ratio 1 ± 0.13.

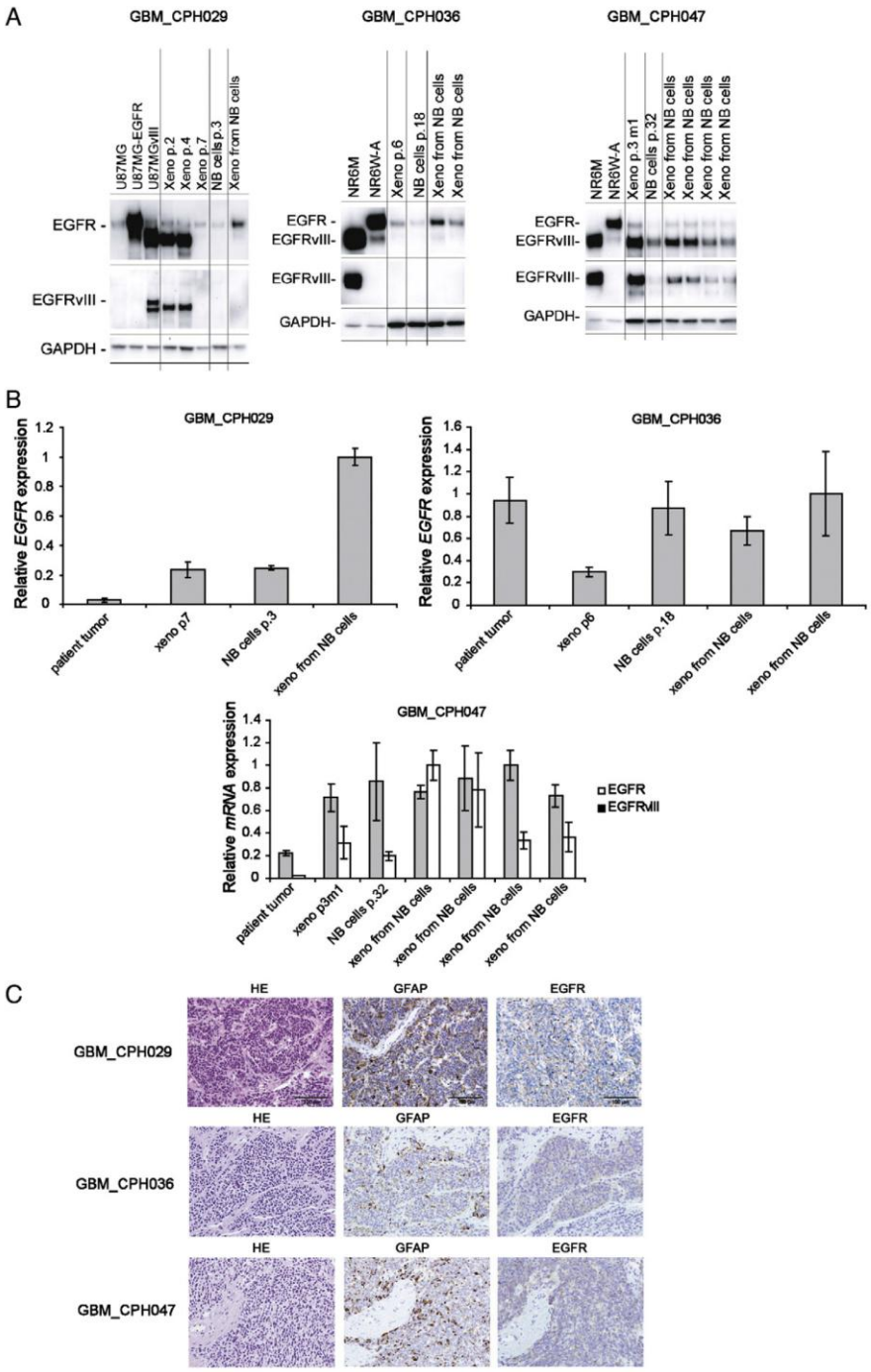
cell culture and in the tumor xenografts (Figs. 7A and B). The NGBM_CPH036p6 xenograft, the derived *in vitro* culture and there of derived secondary xenografts all expressed EGFR, whereas they were negative for EGFRvIII (Figs. 7A and B). Most importantly, all secondary xenografts derived from the NGBM_CPH047p3m1 *in vitro* culture expressed EGFRvIII, as did the original xenograft from which the cells were established (Figs. 7A and B). All samples analyzed from NGBM_CPH047p3m1 also expressed wild-type EGFR (Figs. 7A and B). IHC staining confirmed expression of EGFR in the secondary xenograft tumors (Fig. 7C). HE staining and expression of GFAP, as detected by IHC verified tumors of astrocytic origin (Fig. 7C).

Discussion

Here we describe the establishment of an *in vivo* and *in vitro* model of the human brain tumor GBM. In this model, expressions of EGFR and EGFRvIII were maintained both in xenograft tumors growing subcutaneously on mice and in cell cultures established in stem cell conditions. Furthermore, EGFR and EGFRvIII expressions were preserved in xenograft tumors established from *in vitro* cultured cells. We found that EGFR was expressed in 47% of the patients as detected by IHC and 44% of the patients as detected by WB (tumors rated as xx and xxx were included), and as such was representative for what has been found in other studies with GBM [16,18,43]. In some cases the results regarding EGFR expression obtained from IHC and WB analyses were contradictory. This was most probably due to the heterogenic expression of EGFR, which was most evident in tumors expressing medium levels of EGFR. However, this was also apparent in tumor samples from GBM_CPH017, where EGFR expression was detected as high in the diagnostic patient material (unpublished observations) whereas the piece of patient material that was xenografted into nude mice, and resultant xenograft tumors, were low for EGFR expression as detected by IHC. Humphrey et al. reported a similar discrepancy between patient tumor biopsies and derived xenografts, where they found one case to be highly positive for EGFR expression in the biopsy whereas the resultant xenograft tumor was negative [44]. However, in all other cases examined they found that EGFR expression, as detected by IHC, was identical between the biopsies and the derived xenografts, which is in line with our own results. The diagnostic material from GBM_CPH017 was shown to be amplified for the *EGFR* gene by FISH analysis. This is in line with previous data showing that in most cases, overexpression of EGFR is associated with *EGFR* gene amplification [21,36,44]. However, *EGFR* amplification has been found without overexpression of the EGFR protein [21,45]. This would explain the low expression of EGFR as detected by IHC in the presence of *EGFR* amplification in xenograft tumors derived from GBM_CPH017. Nevertheless, *EGFR* mRNA expression in the GBM_CPH017 patient material xenografted into mice and resultant xenograft tumors was comparable with mRNA expression in GBM_CPH029 and GBM_CPH048, which were both found to be amplified for *EGFR*. This indicates that there is a post-transcriptional regulation that is responsible for the low EGFR expression present in GBM_CPH017.

In addition to overexpression of wild-type EGFR, about one third of primary GBM express the tumor specific deletion variant EGFRvIII which lacks the extracellular, ligand binding domain and which shows low but constitutive activation [16–18,43,46,47]. In our study, expression of EGFRvIII was always accompanied by expression of the wild-type receptor, and never occurred *de novo* in xenografts or cell cultures. Even though GBM_CPH047 was negative for EGFRvIII expression in the patient material as assessed by WB, Q-RT-PCR analysis showed presence of *EGFRvIII* transcript. This discrepancy could either be due to differences in sensitivity of the methods or to heterogeneous distribution of EGFRvIII in the tumor. Focal expression of EGFRvIII has been reported by others, and as sampling then could affect EGFRvIII detection, caution should be taken when analyzing tumor biopsies for the expression of EGFRvIII [22,27]. Heterogenic expression of EGFRvIII might also be an obstacle when establishing *in vitro* cultures from EGFRvIII expressing tumors. This was evident in GBM_CPH029, in which all xenograft passages before and subsequent passage 7 were positive for EGFRvIII expression. However, the xenograft tumor analyzed from passage 7 was negative. From this tumor we established a cell culture, which was also negative for EGFRvIII expression. However, EGFRvIII expression was not lost *in vivo*, as subsequent xenograft passages expressed EGFRvIII, indicating that the lack of EGFRvIII expression in passage 7 and the derived cell culture is a consequence of heterogenic EGFRvIII expression and a result of sampling. Previous reports have shown that EGFRvIII expression in most cases is associated with *EGFR* amplification, even though EGFRvIII expression can also occur

Fig. 7 – EGFR and EGFRvIII expressions are maintained in xenografts derived from *in vitro* cultured cells. EGFR and EGFRvIII expressions in patient tumor, primary xenografts, cell cultures and secondary xenografts from GBM_CPH029, GBM_CPH036 and GBM_CPH047 as assessed by A) Western blotting and B) Quantitative Real-time PCR. U87MG is an established glioma cell line, stably transfected with EGFR (U87MG-EGFR) and EGFRvIII (U87MGvIII) respectively. NR6 are mouse fibroblasts expressing EGFRvIII (M), high levels of EGFR (W) and medium levels of EGFR (WA). C) IHC analysis of GFAP and EGFR expressions in secondary xenograft tumors from GBM_CPH029p7NB cells, NGBM_CPH036p6NB cells and NGBM_CPH047p3m1NB cells. HE staining was used to confirm tumor diagnosis. ×400 magnification. For detection of EGFRvIII by Western blotting, the mouse monoclonal anti-EGFRvIII L8A4 antibody was used.


A
GBM_CPH029
GBM_CPH036
GBM_CPH047
U87MG
U87MG-EGFR
U87MGvIII
Xeno p.2
Xeno p.4
Xeno p.7
NB cells p.3
Xeno from NB cells
NR6M
NR6W-A
Xeno p.6
NB cells p.18
Xeno p.3 m1
NB cells p.32
EGFR -
EGFRvIII -
GAPDH -
B
Relative EGFR expression
patient tumor
xeno p7
NB cells p.3
xeno from NB cells
xeno p6
NB cells p.18
Relative mRNA expression
xeno p3m1
NB cells p.32
EGFR
EGFRvIII
C
HE
GFAP
EGFR

without EGFR amplification in a minority of cases [21,22,48]. We observed EGFRvIII expression in xenografts from GBM_CPH029 and GBM_CPH047, which were shown to be amplified for *EGFR* by FISH and harboring gaining of *EGFR* by MLPA analyses respectively. These data are thus in good correlation to previous reports and strengthen the hypothesis that the EGFRvIII mutation occur subsequent to *EGFR* gene amplification [48].

Based on previous results, *EGFR* amplification and EGFRvIII expression has been considered to be *in vivo* phenomenon as these are invariable lost *in vitro* [26,27]. In an extensive study by Pandita and colleagues it was shown that subpopulations of cells with *EGFR* amplification, present in the original patient tumor, were selected for when grown as xenografts [27]. Furthermore, when both wild-type and *EGFRvIII* amplification were present, there was a selection for the mutated version. In our study, we could not detect any consistent increase in neither EGFR nor EGFRvIII expression in the xenografts as compared to the original patient tumor, and in most cases the *EGFR* amplification status in the xenograft tumors was consistent with what was found in the patients. This is also consistent with the study by Humphrey et al., who showed that the *EGFR* amplification pattern observed in GBM biopsies was preserved in derived xenograft tumors [44]. In addition, in our study, both EGFR and EGFRvIII were continuously expressed in xenograft tumors that co-expressed both receptors, even after several passages. However, we did see a selection for a mutated version of EGFR in xenografts derived from GBM_CPH048. Selection for rearranged EGFR genes after several passages in GBM xenografts has been reported previously [49]. However, as the mutated protein in the GBM_CPH048 xenografts appeared already in passage 1, we find it likely that this rearrangement was present, either at undetectable levels or heterogeneously expressed, also in the parental tumor. This mutated EGFR was larger than the wild-type receptor and could involve duplication of the tyrosine kinase (TK) and calcium-mediated internalization (CAIN) domains, as has been shown to occur in human glioma cells [50,51].

To date, only one established glioma cell line with *EGFR* amplification, and none with endogenous EGFRvIII expression, has been reported [52,53]. Even though *EGFR* gene amplification has been shown to be maintained *in vivo*, cell lines established from the same original patient tumors fail to show any *EGFR* amplification [26,27]. Pandita et al., showed loss of amplified *EGFRvIII* in cell cultures established from GBM xenograft tumors harboring this amplification, and thus suggested that there is a selection against EGFRvIII *in vitro* [27]. However, the cells were grown in traditional media in the presence of serum. This could be of importance when considering a stem cell origin of GBM, as NSC are known to differentiate upon serum exposure [54,55]. Indeed, glioma cell lines established in the presence of serum have been shown to be less representative for the original patient tumor than cell lines established in so-called stem cell conditions [33]. As such, it is possible that stem cell-associated characteristics, important for GBM tumorigenicity, could be lost in traditional, serum-containing culture conditions. One could speculate that EGFR and EGFRvIII expressions might be examples of such characteristics, and preliminary data from our own laboratory strengthen this hypothesis (unpublished data). In the postnatal brain, EGFR is expressed in neurogenic regions also containing neuronal precursor cells [56]. Holland and co-workers have reported the importance of EGFR signaling in the genesis of gliomas from immature cells of the brain [57]. They showed that when introducing constitutively active mutant EGFR into glial precursor cells or astrocytes under the Nestin- and GFAP promoters respectively, lesions with similarities to human gliomas were induced. This occurred more frequently when mutant EGFR was expressed from the Nestin promoter than from GFAP, indicating that the tumors arose more efficiently from immature cells of the glial lineage than from mature astrocytes [57]. Furthermore, activated EGFR signaling, through overexpression of EGFR or EGFRvIII, in post-natal murine neural stem cells (NSC) led to enhanced proliferation, survival and blocked neural differentiation, and EGFRvIII expression promoted a glial cell fate [58]. This is consistent with the hypothesis that stem cell-like cells are involved in the genesis of gliomas and that EGFR signaling is involved in this process.

By culturing GBM cells in stem cell conditions, we could show that EGFR expression was maintained at the same level as in the xenografts from which the cells were derived. Furthermore, gain of *EGFR*, possibly reflecting *EGFR* amplification, as detected by MLPA, was present in the *in vitro* cultured cells. However, it did seem as if the ratio of *EGFR* was lower in the cell cultures than in the tumor xenografts, although this did not have a substantial effect on EGFR transcript or protein expression. This is in contrast to previous results showing that *EGFR* mRNA expression was lower in *in vitro* cultured cells than in the xenografts from which they were derived [26]. This loss of EGFR expression has furthermore been suggested to be linked to loss of tumorigenicity [26,27]. In our study, *in vitro* cultured cells grown in stem cell conditions, were able to form tumors in immunodeficient mice and as such still maintained their tumorigenic potential. In addition, xenografts derived from the *in vitro* cultured cells seemed to have higher EGFR protein expression than the original tumor xenograft, indicating that the capability to express EGFR at high levels is preserved *in vitro*, even though its expression might be dependent upon additional factors present *in vivo*. However, this is purely speculative and needs further clarification. Most importantly, when grown as neurospheres in stem cell conditions, EGFRvIII expression was maintained. Furthermore, when these cells were used for subcutaneous transplantation into nude mice, the resulting xenograft tumors also expressed EGFRvIII. Thus, when establishing *in vitro* cultures of glioma cells it is crucial to use culturing conditions that favor the growth of tumor bCSC, and which will maintain the geno- and phenotype of the original tumor.

In conclusion, we have established xenograft tumors and cell cultures from human GBM that maintain the expressions of EGFR and EGFRvIII. In this *in vivo*/*in vitro* model, it will be possible to further investigate the functional role of these receptors, and response to targeted therapy, in GBM malignancy.

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5. Results - Manuscript II

Differentiation of human glioblastoma multiforme stem-like cells leads to down regulation of EGFR and EGFRvIII expression and decreased tumorigenic and stem-like cell potential

Running title: EGFR/EGFRvIII expression is lost upon differentiation

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Abstract

Glioblastoma multiforme (GBM) is the most common and devastating primary brain tumor among adults. Despite recent treatment progress, most patients succumb to their disease within 2 years from diagnosis. Current research have highlighted the importance of a subpopulation of cells, assigned brain cancer stem-like cells (bCSC), to play a pivotal role in GBM malignancy. bCSC are identified by their resemblance to normal neural stem cells (NSC), and it is speculated that the bCSC have to be targeted in order to improve treatment outcome for GBM patients. One hallmark of GBM is aberrant expression and activation of the epidermal growth factor receptor (EGFR) and expression of a deletion variant EGFRvIII. In the normal brain, EGFR is expressed in neurogenic areas where also NSC are located and it has been shown that EGFR is involved in regulation of NSC proliferation, migration and differentiation. This led us to speculate if EGFR and EGFRvIII are involved in the regulation of bCSC. In this study we use GBM neurosphere cultures, known to preserve bCSC features. We demonstrate that EGFR and EGFRvIII are down regulated upon differentiation and moreover that when EGFR signaling is abrogated, differentiation is induced. Furthermore, we show that differentiation leads to decreased tumorigenic and stem cell-like potential of the neurosphere cultures and that by specifically inhibiting EGFR signaling it is possible to target the bCSC population. Our results suggest that differentiation therapy, possibly along with anti-EGFR treatment would be a feasible treatment option for patients with GBM, by targeting the bCSC population.

Introduction

Glioblastoma multiforme is the most common and aggressive solid tumor occurring in the brain of adults. Despite progress in recent years, the median survival after diagnosis remains around 15 months(1). There are some molecular hallmarks of GBM, of which amplification and/or mutation of the epidermal growth factor receptor (EGFR) belong to the most common. The most frequent mutation is the vIII variant (EGFRvIII) which arises from deletion of exons 2-7, rendering the receptor constitutively active and unable of ligand binding(2). Both over expression of wild-type EGFR and expression of EGFRvIII have been linked to a more aggressive phenotype and dismal prognosis of GBM(3-6). However, even though EGFR and variants thereof are believed to play a role in GBM tumorigenicity, clinical trials with EGFR inhibitors have shown inconsistent results(7-9). Therefore, a deeper knowledge regarding the functional role(s) of EGFR and EGFRvIII in GBM is needed. Until recently, a major obstacle in the EGFR/EGFRvIII research has been that endogenous EGFR over expression and EGFRvIII expression are lost when GBM cells are grown during traditional serum-containing *in vitro* conditions(10;11). However, in a previous publication from our laboratory we showed that GBM cell cultures established during serum-free stem cell conditions, traditionally used for *in vitro* growth of neural stem cells (NSC), maintained endogenous expression of EGFR and EGFRvIII(12). This is in line with other reports showing that GBM cultures grown during stem cell conditions maintain the geno- and phenotypes of the original tumor better than GBM cells cultured during serum-containing conditions(13). These stem cell conditions are believed to preserve and promote growth of so-called brain cancer stem-like cells (bCSC), a population of cancer cells found in GBM which share characteristics with normal NSC. bCSC are defined by their self-renewing potential, their expression of stem cell markers and their capacity to give rise to cells of the three neural lineages, namely astrocytes, oligodendrocytes and neurons, upon differentiation(14-18). The bCSC have been assigned a role in tumor angiogenesis and treatment resistance, and upon intra cranial transplantation onto immunocompromised mice, it has been shown that is the bCSC that are responsible of forming tumors *in vivo*(19-22). Thus it is likely that the bCSC are involved in the initiation and progression of brain tumors such as GBM and that treatment directed against the bulk of the tumor cells fails to give long term responses because the bCSC are unaffected and able to recapitulate the tumor. There is thus a rationale for using stem cell established cultures in experimental GBM research, in order to better understand factors that are important for the bCSC and GBM maintenance.

As mentioned above, bCSC are defined by their self-renewing capacity and their ability to differentiate. These are features that potentially could be used in differentiation based cancer therapy targeting bCSC, as differentiation might lead to hampered self-renewing capacity and reduced production of progenies that are able to populate the tumor. One example of this is acute promyelocytic leukemia (APL) in which poorly differentiated leukemia cells populate the bone marrow and blood, and compete with the production of normal blood cells. Upon induced differentiation therapy with *all*-trans-retinoic acid (RA), these immature cells are forced to differentiate and thereby lose their malignant potential(23;24).

In this study, we investigated the impact of induced differentiation on the endogenous expression of EGFR and EGFRvIII in human derived GBM neurosphere cultures in order to elucidate their roles in GBM tumorigenicity.

Materials and methods

Cell culture and reagents

Establishment and characterization of the human derived GBM xenograft (NGBM_CPH047) and the *in vitro* GBM cell culture (NGBM_CPH047p3m1) used in this study has been previously described and show endogenous EGFRvIII expression(12). All cells were maintained as neurosphere cultures in Neurobasal®–A media (NB) (Invitrogen, Taastrup, Denmark) with the following additives: N2, B27, bFGF (10ng/ml), EGF (10ng/ml), L-glutamine, penicillin (50U/ml) and streptomycin (50µg/ml) (all from Invitrogen, Taastrup, Denmark), in an atmosphere of 5% CO_2 and 21% O_2 at 37°C. Fresh media was added twice a week and spheres were mechanically dissociated at every passage. For experiments, cells were dissociated, counted and plated in NB media plus all additives as above or in DMEM with the addition of FCS (10%), penicillin (50U/ml) and streptomycin (50µg/ml) prior to initiation of treatment with *all*-trans-retinoic acid (RA) (10µM, Sigma-Aldrich, Broendby, Denmark) or AG1478 (1, 5 or 10µM, Calbiochem, Hellerup, Denmark) for 12 to 14 days. In control experiments DMSO was added at the same volume as the drugs, if not stated otherwise.

Primary sphere assay

Single cells from acutely dissociated GBM xenograft tissue (NGBM_CPH047) were plated in 96-well microwell plates at a density of 10 cells/µl and directly treated with 0 or 5µM AG1478. 0µM AG1478 was used as a control in this assay. At day 14, the number of spheres per well was scored and the primary sphere frequency was calculated.

Western Blotting

Whole cell protein lysates were separated on 3-8% NuPAGE TA gels or on 4-12% NuPAGE Bis-Tris gels (Invitrogen, Taastrup, Denmark) and electroblotted onto nitrocellulose membranes (Invitrogen, Taastrup, Denmark). The membranes were then blocked for 1 hour (hr) at room temperature (RT) and incubated with primary antibodies in 5% non-fat milk overnight (ON) at 4ºC followed by secondary antibodies for 1 hr at RT. Blots were developed using the SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL) and the Biospectrum Imaging System (UVP, Upland, CA). Primary antibodies used: goat polyclonal anti-EGFR (Fitzgerald Industries International, Concorde, MA), mouse monoclonal anti-EGFRvIII (L8A4, a kind gift from Dr. Darrell Bigner, Duke University Medical Center, Durham, NC), mouse

monoclonal anti-GFAP (Cell Signaling, Boston, MA), rabbit polyclonal anti-EGFR [pY^{1086}] (Biosource, Invitrogen, Carlsbad, CA) and rabbit anti-GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Quantitative Real-time PCR (Q-RT-PCR)

GBM neurosphere cultures were harvested, quick frozen in liquid N_2 and stored at –80°C until used. Total RNA was purified with the RNeasy Mini kit and QIAshredder (both from Qiagen Denmark, Copenhagen, Denmark) as described by the manufacturer. All RNA was DNase treated using the RNase-Free DNase Set from Qiagen Denmark (Copenhagen, Denmark) according to the manufacturer's instructions. cDNA was synthesized using the SuperscriptTM III Platinum® Two step qRT-PCR kit with SYBR® Green (Invitrogen, Taastrup, Denmark) which was also used for quantitative real-time PCR (Q-RT-PCR) reactions. Relative quantification of gene expression levels was performed according to the comparative Ct method. All data was normalized to the expression of three housekeeping genes (*TOP1*, *EIF4A2* and *CYC1*) included in the human geNorm house-keeping gene selection kit (Primerdesign, Southampton, UK). PCR reactions were optimized to distinguish between *EGFR* and *EGFRvIII* expression as previously described(12). Primers used were: *EGFR* forward, 5' –TCC TTG GGA ATT TGG AAA TT- 3'; *EGFR* reverse, 5' –GGC ATA GGA ATT TTC GTA GTA CAT- 3'; *EGFRvIII* forward, 5' –ATG CGA CCC TCC GGG ACG- 3'; *EGFRvIII* reverse, 5' –ATC TGT CAC CAC ATA ATT ACC T- 3' [16]; *GFAP* forward, 5' -CGC TGG TAG AGA TGG AGG AG- 3'; *GFAP* reverse, 5' -CTG GGG TTA AGA AGC AGC AG- 3'.

MTT

MTT assays were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay according to the manufacturer's instructions (Sigma, Broendby, Denmark). Briefly, neurospheres were dissociated and cells were plated in a 96-well cell culture plate at a density of 8 x 10^4 cells per well and allowed to grow ON. Drugs were added at the indicated concentrations where after the plates were incubated for 12 days at 37°C, 5% CO_2, 21% O_2. At the end of experiment, 20µl 5mg/ml MTT solution (dissolved in sterile water) was added to each well and incubated for 4 hours before addition of 100 µl solubilization buffer (10% SDS, 0.03M HCl). Next day, the absorbance was read at 570nm using a Synergy2 microplate reader with the Gen5, Microplate Data Collection & Analysis Software (Biotek, Winooski, Vermont, USA). A reference

filter at 690nm was used to subtract background absorbance. Each experimental condition was tested in sextuplicates and repeated at least two times.

Soft Agar

Dissociated neurosphere cells (1 x 10^5/ml) were casted in semisolid agar with either DMEM + 10% FCS or NB media including all supplements as described above, in the presence of either RA (10µM) or AG1478 (5µM or 10µM). At day 14, the colonies were stained with a 0.005% Crystal Violet solution and the number of colonies was manually counted.

Sub-sphere analysis

Neurospheres were mechanically dissociated and seeded at a density of 1 x 10^6 cells in either DMEM + 10% FCS or NB media including all supplements as described above and allowed to grow ON. RA (10µM) or AG1478 (5 or 10µM) or the corresponding volume DMSO as a control was added to the cells, which were then incubated for 12 days for pre-treatment. Adherent and suspension cells were then collected by centrifugation, dissociated and diluted in NB media with all additives. 10 or 100 cells/well were then plated in 96-well plates and allowed to form spheres. The total number of spheres was counted manually after 2 weeks.

Results

Serum and RA induce differentiation of human GBM cells

In this study we have used a GBM cell culture established during stem cell conditions in the absence of serum. In this culture system the cells grow as neurospheres and share characteristics with normal NSC(25). We have recently shown that such cultures maintain important features of GBM such as expression of EGFR and EGFRvIII(12), whereas other studies have shown that GBM cells grown during traditional cell culture conditions in the presence of serum fail to maintain EGFR and EGFRvIII expression(10;11). In addition, it has been shown that bCSC are able to differentiate *in vitro* upon serum exposure(17). As a first experiment we therefore wished to study the effect of serum on our GBM neurosphere culture with endogenous EGFR and EGFRvIII expression. Upon serum exposure, some, but not all, neurospheres became adherent and cells started to migrate out from the spheres (Figure 1A). The adherent cells grew with a differentiated phenotype, with neurite-like extensions. Q-RT-PCR analysis confirmed up regulation of the astrocytic marker glial fibrillary acidic protein (GFAP) (Figure 1B), verifying that serum exposure indeed induces differentiation of GBM neurosphere cells. As an additional differentiation agent, RA has in previous studies been shown to induce differentiation of GBM neurosphere cells *in vitro*(26;27). Indeed, RA induced both morphological differentiation and up regulation of GFAP mRNA in our cells, although not as effectively as serum (data not shown and Figure 1C).

EGFR and EGFRvIII expression are lost upon induced differentiation

Having verified that serum and RA induce differentiation of our neurosphere cells, we investigated the impact of differentiation on EGFR and EGFRvIII expression. For this we grew the cells in serum containing media or treated them with RA in NB media. We first examined the mRNA level of both EGFR and EGFRvIII. As shown in Figure 2A and 2B, both EGFR and EGFRvIII mRNA were down regulated upon induced differentiation with either serum or RA. Furthermore, differentiation led to down regulation of both EGFR and EGFRvIII protein expression (Figure 2C). Although RA was not as effective in inducing GFAP up regulation as serum (Figure 1C and Figure 2C), there was no major difference in EGFR and EGFRvIII down regulation (Figure 2C).

Inhibition of EGFR signaling leads to differentiation of GBM cells and reduced cell viability

The above results led us to speculate if EGFR and EGFRvIII signaling were coupled to a less differentiated cell phenotype and that these receptors could be involved in maintaining an

undifferentiated pool of neurosphere cells. Therefore, we inhibited EGFR/EGFRvIII signaling with the tyrosine kinase inhibitor (TKI) AG1478. Exposure to AG1478 led to decreased phosphorylation of EGFR (pY1086) (Figure 3A) along with reduced number of viable cells (Figure 3B), smaller neurospheres in culture and in some cases also adherent and morphologically differentiated cells (Figure 3C and data not shown). In addition, treatment of GBM neurosphere cells with AG1478 led to up regulation of GFAP mRNA (Figure 3D) and protein in a concentration dependent manner (Figure 3E), indicating that active EGFR/EGFRvIII signaling is involved in maintaining a less differentiated cell phenotype.

Induced differentiation or inhibition of EGFR signaling leads to decreased tumorigenicity and reduced stem cell potential

Having verified that differentiation leads to down regulation of EGFR/EGFRvIII and that inhibition of EGFR/EGFRvIII signaling with AG1478 leads to differentiation, we wished to investigate which impact EGFR/EGFRvIII had on the tumorigenic capacity and stem cell potential of our GBM cells. Therefore, we performed a soft agar assay, reflecting the *in vitro* tumorigenic potential of the cells. Here, it was evident that both induced differentiation with RA and inhibition of EGFR/EGFRvIII with AG1478 led to decreased ability to grow without anchorage as measured by reduced colony formation (Figure 4A). Although serum alone did not reduce colony formation, the colonies were clearly smaller than colonies formed in NB media alone (Figure 4B). In addition, when serum was combined with RA colony formation was clearly reduced, both in comparison to colonies formed in NB media alone and in comparison to colonies formed in the presence of RA in NB media (Figure 4A). In a sub-sphere assay, reflecting the number of cells with self-renewing potential, i.e. an indirect measurement of the number of stem cells present, the capacity to form spheres was markedly hampered after induced differentiation with serum or inhibition of EGFR/EGFRvIII signaling with AG1478 (Figure 4C). However, sub-sphere formation after pre-treatment with RA in NB media was increased as compared to NB media alone and spheres formed were also larger (Figure 4C and data not shown).

It is considered that the number of bCSCs in a primary culture, i.e. the first passage when a tumor is established as an *in vitro* culture, can be identified by their ability to form neurospheres during clonogenic dilution(28;29). Therefore the fraction of GBM cells forming spheres in the primary culture can be interpreted as a semi-quantification of the bCSC population in the tumor. To investigate the impact of EGFR/EGFRvIII on primary neurosphere formation and thus indirect the

effect on the bCSC population we therefore performed a primary sphere assay in the presence of AG1478. As shown in Figure 4D, our results indicate that upon AG1478 exposure, primary sphere formation was significantly reduced.

In conclusion, our data together imply that functional EGFR/EGFRvIII signaling is important for maintaining an undifferentiated cell phenotype and bCSC potential in GBM neurosphere cultures.

Discussion

Here we show that induced differentiation with serum or RA led to down regulation of EGFR and EGFRvIII expression in human GBM. Furthermore, when inhibiting EGFR and EGFRvIII signaling with an EGFR specific TKI, differentiation was induced. In addition, down regulation of EGFR and EGFRvIII, as a result of induced differentiation or inhibition of their signaling, led to decreased *in vitro* tumorigenic capacity and reduced bCSC potential of the GBM cells.

Over the last decade, several studies have identified a population of cells in GBM, with resemblance to normal NSC regarding self-renewal and multi differentiation potential and therefore designated bCSC(14-18). These bCSC have been suggested to be responsible for treatment resistance and recapitulating tumor growth in GBM patients(13;14;16;18-22). However, the question regarding how to identify and target the bCSC still remains unanswered. The CD133 marker has gained much attention as a suitable bCSC marker, yet several studies have now shown that also cells without CD133 expression have stem cell potential and are able to form tumors *in vivo* and also that there are several distinct pools of bCSC that coexist within the same tumor(30;31). Nevertheless, even if the exact molecular profile of bCSC in GBM is presently unknown, it is likely the bCSC that have to be targeted in order to inhibit tumor growth, and especially remission. Therefore it is of importance to identify factors that are involved in bCSC maintenance and as such could be used for bCSC directed cancer therapy. The cell of origin for bCSCs still needs to be elucidated. Clinically, most GBM are located in association to neurogenic areas of the brain such as the subventricular zone (SVZ) lining the lateral ventricles and in the dentate gyrus of the hippocampus. It is also in these areas where normal NSC are most abundant(32;33), and it is therefore not farfetched to speculate that these cells could be involved the genesis of bCSC and tumor initiation(32;34). Furthermore, EGFR is expressed in neurogenic regions of the brain such as the SVZ(35), and it has been shown that EGFR is involved in the regulation of NSC proliferation, differentiation and migration(36-38). It is thus likely that EGFR also plays a role in bCSC. We have in a recent study shown that expression of EGFR and especially EGFRvIII is maintained in GBM neurosphere cultures in the presence of EGF and bFGF(12), and others have shown that such cultures promote and preserve growth of bCSC(13). It is, furthermore, well known that GBM cells lose the expression of EGFRvIII and over expression of EGFR in cultures established under *in vitro* growth conditions which contain serum(11). Our results here show that when GBM neurosphere cultures are grown in the presence of serum, they are induced to differentiate, as shown by a prominent up regulation of GFAP. This is also in line with other studies

showing that serum cultured neurosphere cells express more GFAP and Tuj1 as compared to their NB cultured counterparts(13). These data imply that when grown in the presence of serum, differentiation of the GBM neurosphere cells is stimulated. Along with differentiation, we observed a down regulation of EGFR and EGFRvIII, both at mRNA and protein levels. This led us to the conclusion that when GBM cells are grown in the presence of serum, EGFR and EGFRvIII expression are lost due to induced differentiation, and that this might explain the previous lack of success of establishing GBM cultures with endogenous EGFR and EGFRvIII expression as the majority of studies have used serum containing *in vitro* conditions(11).

RA has been shown to induce GBM neurosphere cell differentiation *in vitro* as shown by changes in morphology and up regulation of differentiation markers(26;27). Treatment with RA in our study also resulted in morphological differentiation and up regulation of GFAP. However, the increase in GFAP expression was not as prominent as after serum exposure. This is in contrast to what was shown by Campos *et al.*, who showed that RA was a better inducer of astrocytic differentiation than serum(26). Despite these differences, we concluded that RA induces differentiation of GBM neurosphere cells. As with serum induced differentiation, exposure of GBM neurosphere cells to RA resulted in down regulation of both EGFR and EGFRvIII expression.

EGFR and EGFRvIII have been coupled to a cancer stem-like cell (CSC) phenotype. For example, EGFR knock-down in EGFR positive GBM neurosphere cultures led to differentiation and less malignant tumors *in vivo*(39) and in another study EGFR inhibition resulted in reduced sphere formation in the presence of EGF in EGFR positive neurosphere cultures(40). In breast cancer, EGFRvIII has been shown to contribute to the CSC phenotype as it was correlated to increased expression of stem cell related genes in primary breast cancer samples and led to increased sphere formation *in vitro* and tumor formation *in vivo*(41). Furthermore, in GBM it has been demonstrated that EGFRvIII is co-expressed with CD133 and that these EGFRvIII/CD133 expressing cells have elevated sphere forming capacity(42). Previous studies have shown that differentiation induced by RA targets bCSC in GBM as shown by down regulation of stem cell markers, decreased colony formation in soft agar and a decreased number of CD133 positive cells(26;27). Our results here show that induced differentiation of GBM cells *in vitro* led to down regulation of EGFR and EGFRvIII along with decreased ability of anchorage independent growth, colony formation in soft agar, and sub-sphere formation, indicating that both EGFR and EGFRvIII are involved in bCSC maintenance. However, in these assays there were some differences regarding the effect of serum- and RA induced differentiation. In the soft agar assay, RA was a potent inhibitor of colony

formation, whereas it actually increased both the number of spheres formed and their size in the sub-sphere assay. On the contrary, serum failed to inhibit colony formation in the soft agar assay, although colonies formed were clearly smaller than colonies formed in the control. Furthermore, in the sub-sphere assay serum exposure led to a decrease in the sphere forming potential. One major difference between the two assays performed in this study is that in the sub-sphere assay, differentiation was induced prior to formation of spheres whereas in the soft agar assay the cells were treated while forming colonies. As such, the outcome on sphere- and colony formation cannot be entirely compared. Still, the differences might be explained by the magnitude of differentiation, and as such it seems as if RA is a weaker inducer of differentiation than serum (Figure 5). If RA targets bCSC to differentiate into progenitor cells that are able of limited self-renewal but proliferate faster(43) this would explain the increased sphere number and size in the sub-sphere assay when RA treatment is relieved and also the low GFAP expression observed after induced differentiation. On the other hand serum might induce a close-to terminal differentiation of bCSC or progenitor cells which explains the higher GFAP expression and decreased sphere number in the sub-sphere assay after dissociation. Still, these cells might retain some tumorigenic potential, although not stem cell characteristics, and as such can form colonies in soft agar. However, one could speculate that upon dissociation these cells would not be able to form new colonies or spheres, as was the result in the sub-sphere assay.

If EGFR and EGFRvIII are important for bCSC maintenance in GBM, one could speculate that it would be possible to target bCSC by inhibiting EGFR and downstream signaling, and that such inhibition would result in differentiation of the bCSC and thus reduced bCSC and tumorigenic potential. Soeda *et al*. showed that EGF addition increased the CD133-positive population in bCSC cultures and that this increase could be inhibited by blockage of EGFR signaling(44). In addition, in a recent study it was shown that when inhibiting EGFR/EGFRvIII signaling with AG1478 in breast cancer cell lines transfected with EGFRvIII, the fraction of aldefluor-positive cells, as a read out for CSC, was decreased(41). Indeed, when we inhibited EGFR/EGFRvIII signaling with AG1478, a concentration dependent up regulation of GFAP was observed indicating increased differentiation. Again, the up regulation was smaller than with RA and serum. There are, however, several explanations to this. First, the cell cultures we investigate most probably only contain a minority population of bCSC. Second, it is most likely that there are several groups of different types of bCSC and they might not all be equal regarding their molecular profiles(31). This has also been shown in breast cancer where EGFRvIII was expressed in a subset of the CSC(41). As such, EGFR

and EGFRvIII might not be expressed on all bCSC present. Therefore, when targeting the bCSC through EGFR and EGFRvIII, the molecular effect might not be as substantial as when differentiation is induced by serum or RA, which targets all the cells in the culture. Even though, we still observed an effect on cell viability and *in vitro* tumorigenic potential from AG1478 treatment. Furthermore, as an indirect quantification of the bCSC population, inhibition of EGFR/EGFRvIII with AG1478 in both the sub-sphere and primary sphere assays resulted in a significantly reduced sphere forming capacity. This indicates that EGFR/EGFRvIII are involved in bCSC maintenance and that it would be possible to target bCSC that express EGFR/EGFRvIII through inhibition of EGFR signaling. However, as discussed above, bCSC only constitute a fraction of the total number of tumor cells, and it is likely that not all bCSC express EGFR or EGFRvIII. It has recently been shown that GBM tumors can be divided into several sub groups depending on gene expression, possibly reflecting tumor origin(45;46). Therefore we believe that it is of importance to identify other potential therapeutic targets, specific for each tumor sub group and other bCSC subsets, and use these in combination with anti-EGFR therapy, along with differentiation inducing agents, in the treatment of GBM.

In conclusion, based the presented results vi suggest that active EGFR/EGFRvIII signaling plays an important role for maintaining the stem cell features of a subset of bCSC in EGFR/EGFRvIII positive GBM and for these cells to uphold their tumorigenic potential.

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Figure Legends

Figure 1. GBM neurosphere cells can be induced to differentiate *in vitro*. A) GBM neurosphere cells grow with a differentiated morphology upon serum exposure. Scale bar shows 100µm. B) GFAP mRNA is up regulated in GBM neurosphere cells when grown in serum containing media. C) RA induces up regulation of GFAP mRNA in GBM neurosphere cells. Q-RT-PCR reactions are presented as mean mRNA expression ± SD. Statistical significance was calculated using Student's two-sided t-test. *** = $p<0.005$.

Figure 2. EGFR and EGFRvIII are down regulated in GBM neurosphere cells upon differentiation. Q-RT-PCR analyses showing down regulation of EGFR and EGFRvIII mRNA in GBM neurosphere cells upon A) serum exposure and B) RA treatment. Q-RT-PCR reactions are presented as mean mRNA expression ± SD. Statistical significance was calculated using Student's two-sided t-test. *** = $p<0.005$ and ** =$p<0.01$. C) WB showing down regulation of EGFR and EGFRvIII protein after induced differentiation with serum or RA as shown by up regulation of GFAP.

Figure 3. Abrogation of EGFR signaling with the TKI AG1478 reduces cell viability and leads to induced differentiation. A) WB showing reduced phosphorylation of EGFR upon AG1478 treatment. B) AG1478 (10µM) exposure leads to a decrease in the number of viable cells as measured by MTT. C) Photographs of GBM neurospheres showing reduced sphere size upon AG1478 treatment. Scale bar shows 100µm. D) Bar chart showing GFAP mRNA up regulation by Q-RT-PCR in two independent experiments after AG1478 (10µM) exposure. E) GFAP protein is up regulated in a concentration dependent manner upon AG1478 exposure. Q-RT-PCR reactions and MTT results are presented as mean mRNA expression and viability ± SD respectively. Statistical significance was calculated using Student's two-sided t-test. *** = $p<0.005$ and ** =$p<0.01$.

Figure 4. GBM neurosphere tumorigenicity and stem cell-like potential is reduced upon induced differentiation and after EGFR inhibition. A) Soft agar assay showing reduced colony formation after induced differentiation or AG1478 treatment. Data are presented as mean ± SD of two independent experiments performed in duplicates. B) Representative pictures of soft agar assay using either NB media or DMEM+10%FCS. C) Sub-sphere assay showing reduced sphere forming potential after pre-treatment with serum (DMEM+10%FCS), RA or AG1478 as compared to

spheres formed in NB media only. Data presented are from one out of two independent experiments performed in sextuplicates and are presented as mean number of spheres ± SD. D) Primary sphere formation is reduced upon abrogation of EGFR signaling with AG1478. Data is presented as mean number of spheres ± SD. Statistical significance was calculated between treated and corresponding control, if not indicated otherwise, using Student's two-sided t-test. *** = $p<0.005$, ** = $p<0.01$ and * = $p<0.05$.

Figure 5. Schematic view of proposed serum- and RA induced differentiation of bCSC. Serum induces a close-to terminal differentiation of immature cancer cells such as bCSC and progenitor cells. The resulting cells lose EGFR and EGFRvIII expression while up regulating GFAP expression. However, the cells still retain some tumorigenic potential and proliferate, although they are not able to self-renew. RA induces less differentiation than serum as visible by less up regulation of GFAP. In addition, the cells retain some capacity of self-renewal and proliferation and as such they could represent some stage of progenitor cell differentiation. However, both EGFR and EGFRvIII expression are lost upon differentiation, indicating that these receptors are expressed in an immature and undifferentiated cell type such as the bCSC.

Figure 1

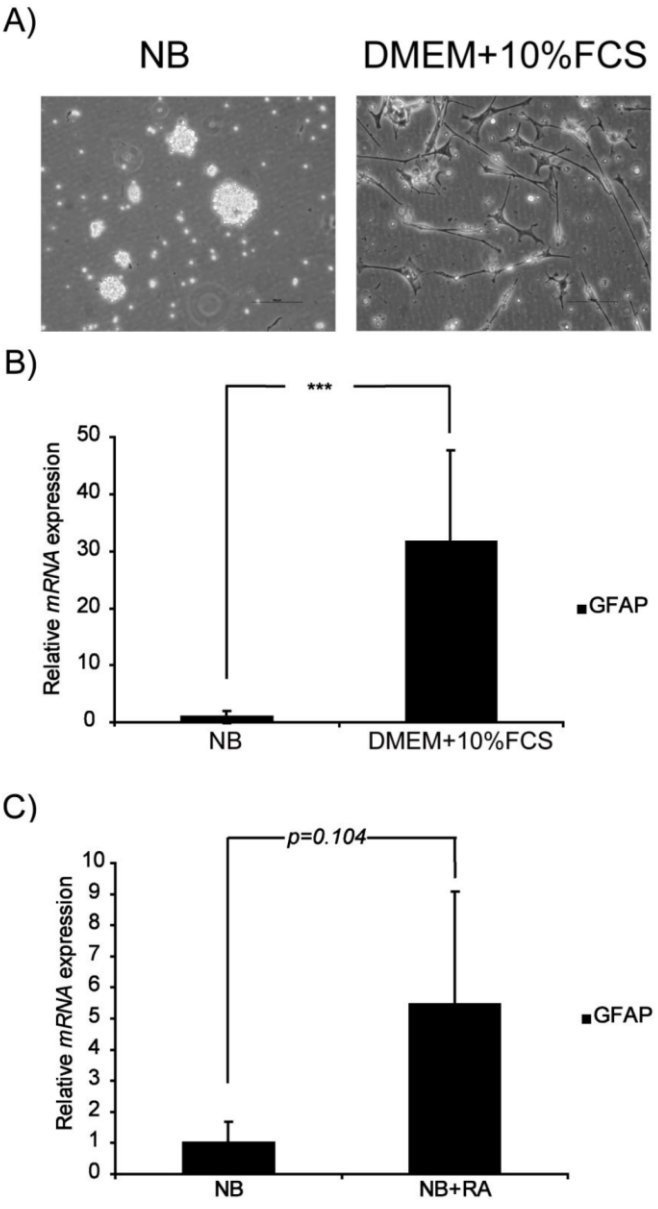

A)
NB
DMEM+10%FCS
B)
***
Relative mRNA expression
50
40
30
20
10
0
NB
DMEM+10%FCS
GFAP
C)
p=0.104
Relative mRNA expression
10
9
8
7
6
5
4
3
2
1
0
NB
NB+RA
GFAP

Figure 2

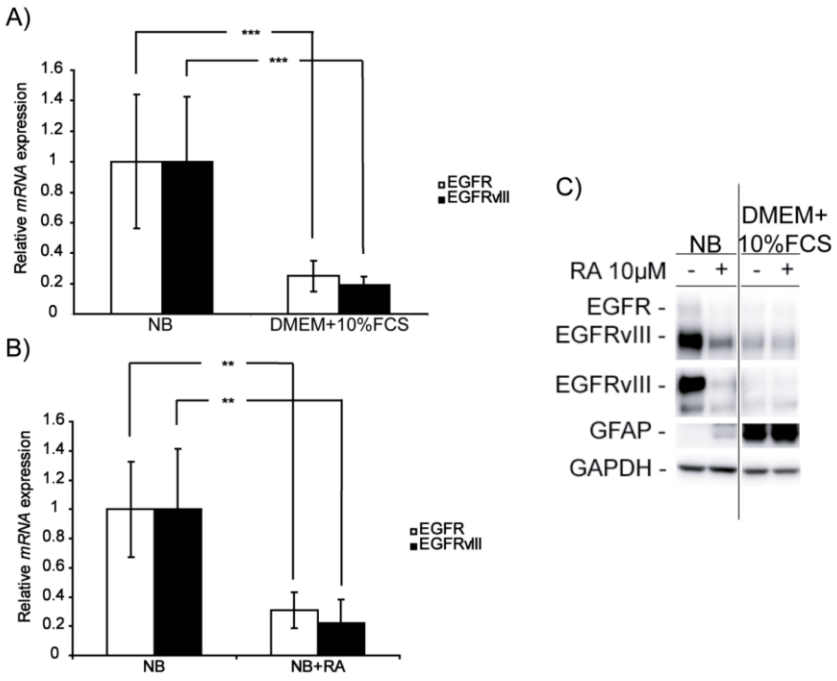

A)
***
***
Relative mRNA expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NB
DMEM+10%FCS
EGFR
EGFRvIII
B)
**
**
Relative mRNA expression
NB
NB+RA
EGFR
EGFRvIII
C)
NB
DMEM+ 10%FCS
RA 10µM - + - +
EGFR -
EGFRvIII -
EGFRvIII -
GFAP -
GAPDH -

Figure 3

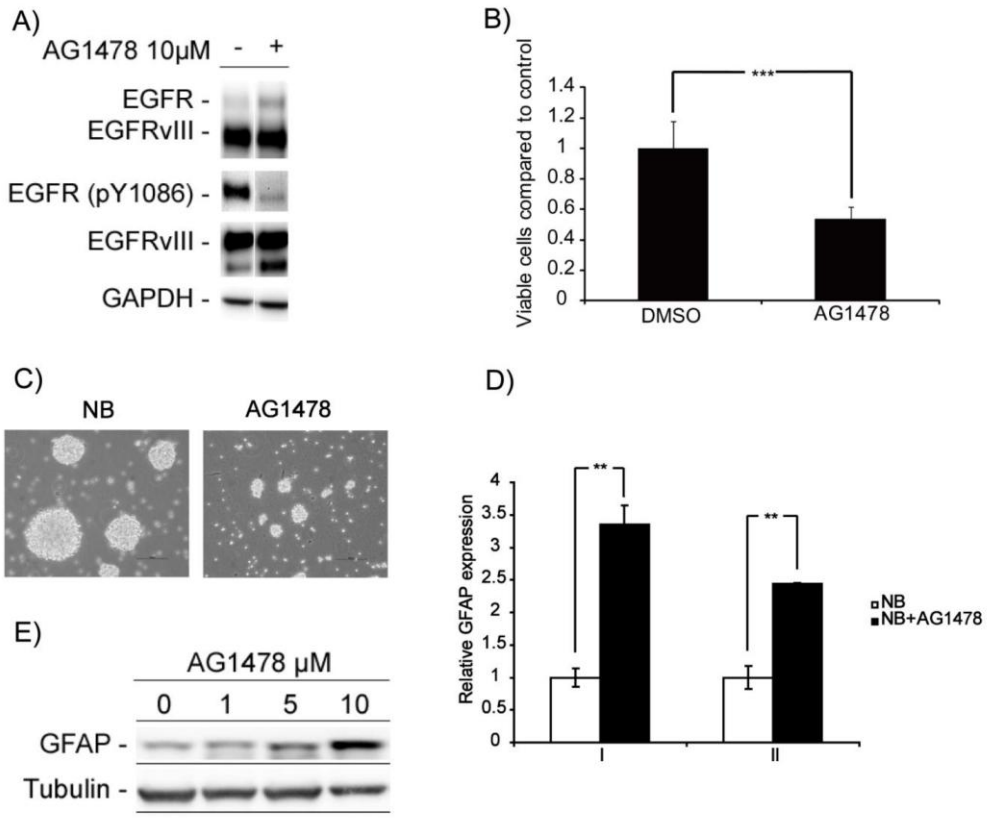

A)
AG1478 10μM - +
EGFR -
EGFRvIII -
EGFR (pY1086) -
EGFRvIII -
GAPDH -
B)
Viable cells compared to control
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
DMSO
AG1478
C)
NB
AG1478
D)
Relative GFAP expression
4
3.5
3
2.5
2
1.5
1
0.5
0
**
**
I
II
NB
NB+AG1478
E)
AG1478 μM
0 1 5 10
GFAP -
Tubulin -

Figure 4

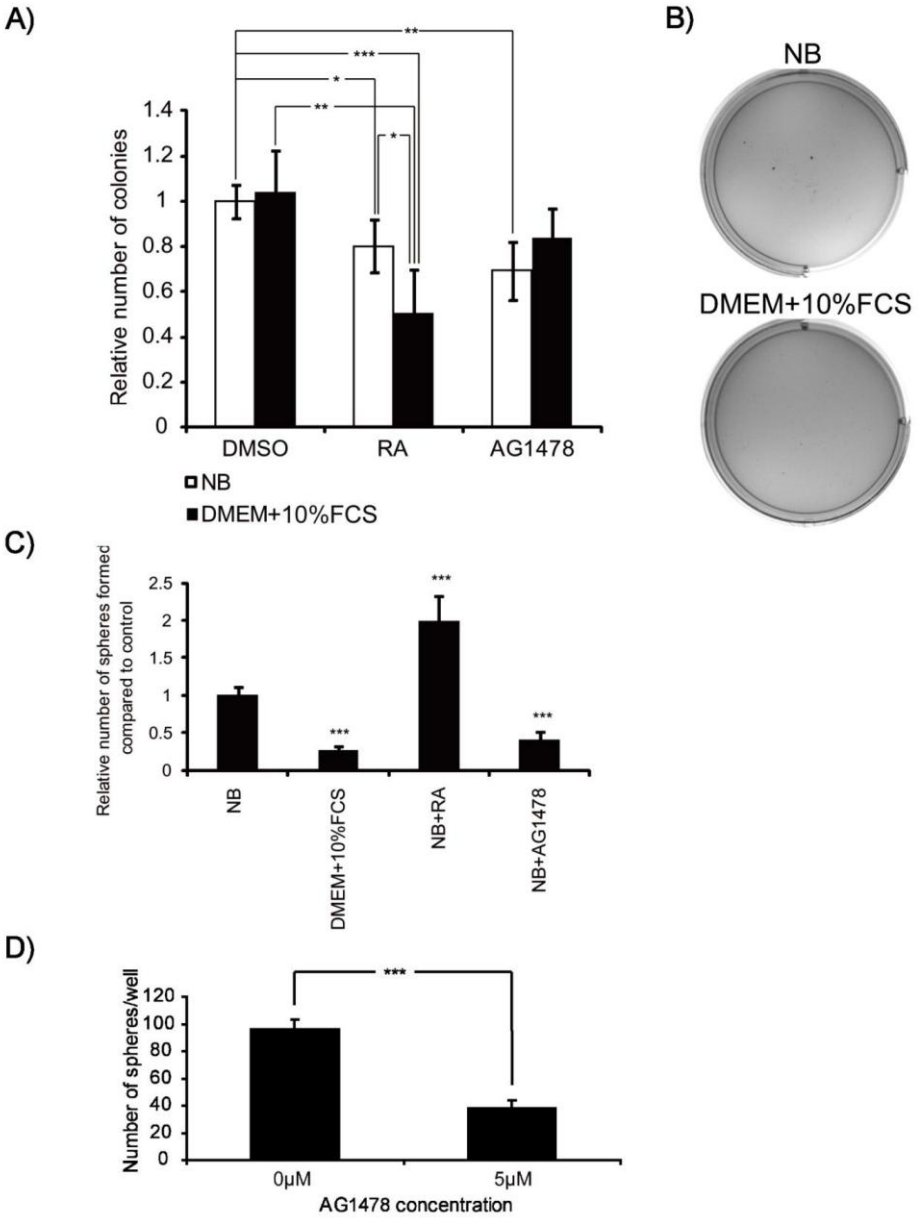
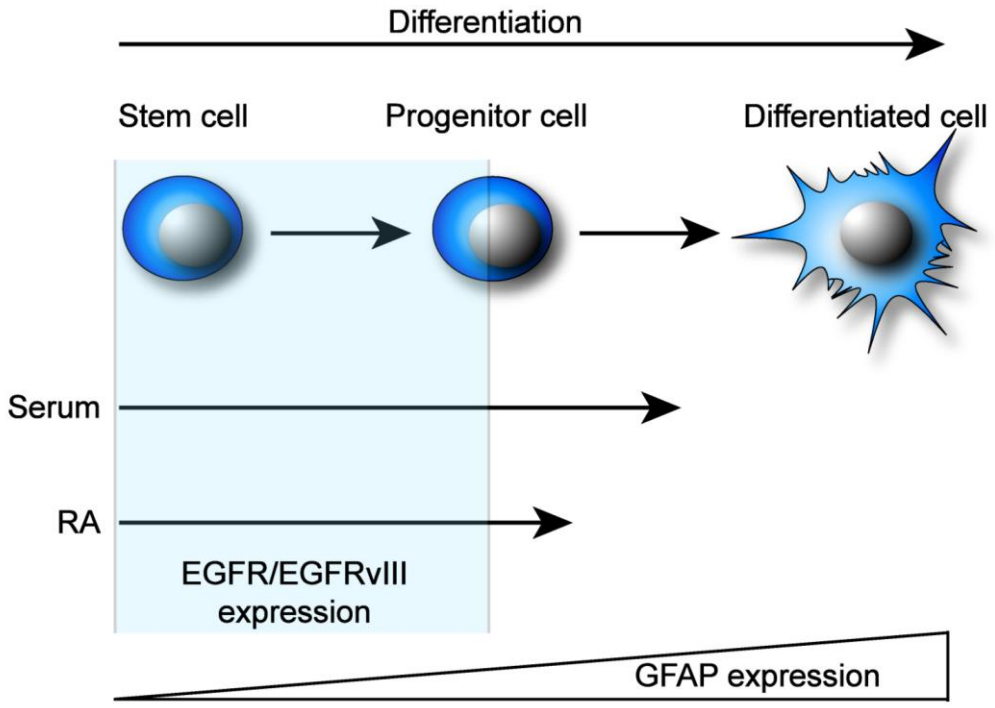

A)
Relative number of colonies
1.4
1.2
1
0.8
0.6
0.4
0.2
0
DMSO
RA
AG1478
NB
DMEM+10%FCS
B)
NB
DMEM+10%FCS
C)
Relative number of spheres formed compared to control
2.5
2
1.5
1
0.5
0
NB
DMEM+10%FCS
NB+RA
NB+AG1478
D)
Number of spheres/well
120
100
80
60
40
20
0
0µM
5µM
AG1478 concentration

Figure 5


Differentiation
Stem cell
Progenitor cell
Differentiated cell
Serum
RA
EGFR/EGFRvIII
expression
GFAP expression

26

6. Results - Manuscript III

Level of Notch activation determines the effect on growth and stem cell-like features in glioblastoma multiforme neurosphere cultures

Running title: Notch signaling in brain cancer stem-like cells

By:

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Level of Notch activation determines the effect on growth and stem cell-like features in glioblastoma multiforme neurosphere cultures

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Keywords: glioblastoma multiforme, neurosphere cultures, brain cancer stem-like cells, Notch signaling, Notch activity, DAPT, ICN-1

Abbreviations: bCSC, brain cancer stem-like cells; DAPT, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-butyl ester; GBM, glioblastoma multiforme; GSI, γ-secretase inhibitor; ICN-1, intracellular Notch-1 domain; NSC, neural stem cells; G_0/G_1, cell cycle phase where the cells are non-dividing

Background: Brain cancer stem-like cells (bCSC) are cancer cells with neural stem cell (NSC)-like properties found in glioblastoma multiforme (GBM) and they are assigned a central role in tumor initiation, progression and relapse. The Notch pathway is important for maintenance and cell fate decisions in the normal NSC population. Notch signaling is often deregulated in GBM and recent results suggest that this pathway plays a significant role in bCSC as well. We therefore wished to further elucidate the role of Notch activation in GBM-derived bCSC.

Results: GBM neurosphere cultures with high endogenous Notch activation displayed sensitivity toward Notch inhibition with regard to tumorigenic features as demonstrated by increased G_0/G_1 population and reduced colony formation capacity. Of the NSC-like characteristics, only the primary sphere forming potential was affected, while no effect was observed on self-renewal or differentiation. In contrast, when Notch signaling was activated a decrease in the G_0/G_1 population and an enhanced capability of colony formation was observed, along with increased self-renewal and de-differentiation.

Methods: Human-derived GBM xenograft cells were cultured as NSC-like neurosphere cultures. Notch modulation was accomplished either by blocking the pathway using the γ-secretase inhibitor DAPT or by activating it by transfecting the cells with the constitutive active Notch-1 receptor.

Conclusion: Based on the presented results we propose that active Notch signaling plays a role for cell growth and stem cell-like features in GBM neurosphere cultures and that Notch-targeted anti-bCSC treatment could be feasible for GBM patients with high endogenous Notch pathway activation.

Introduction

Gliomas are the most common primary brain tumors (PBT) in adults with an yearly incidence of approximately 6/100 000 in Western countries.[1] They are traditionally categorized as derived from glial tissue and further distinction is made based on their grade of anaplasia of which the astrocytic glioma, glioblastoma multiforme (GBM, WHO Grade IV) displays the highest degree. GBM accounts for 50–60% of gliomas[2] and is recognized as the most aggressive PBT in adults with a median survival around 15 mo.[3] The majority of GBMs are often difficult to operate, due to their location and infiltrative growth pattern, and non-surgical treatments (chemo- and radiation therapy) are often ineffective.[4,5] As such, relapse is almost certain, which is why GBM thus far is considered incurable and new treatment modalities are in high demand. Increasing evidence imply that a population of stem-like cells exist within the heterogeneous cell mass that comprise brain tumors, including GBM. These are, among others, designated brain cancer stem-like cells (bCSC) as they show close resemblance to the normal neural stem cells (NSC) of the human brain. The bCSC have been shown to drive tumor initiation and progression in an orthotopic GBM model[6] and they are furthermore thought to exert a significant role in tumor angiogenesis, treatment resistance, and relapse,[7-9] making them an interesting target in the search for improving GBM treatment. Using the serum-free neurosphere culture system and analysis, adopted from work with NSC, bCSC can be identified in vitro based on their NSC-like characteristics, namely, neurosphere formation, self-renewal, multipotency, and expression of NSC markers.[10-14] In addition to NSC characteristics, bCSC are tumorigenic and capable of mimicking the characteristics of the original patient tumor when transplanted onto immunocompromised mice.[12,13,15]

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Results from our lab and others have shown that GBM cell cultures established during stem cell conditions more closely mirror the original patient tumor and maintain important GBM hallmarks, such as amplification and/or mutation of the epidermal growth factor receptor (EGFR),[16,17] than GBM cells grown in the traditional serum containing culture systems. As such, establishing GBM neurosphere cell cultures that retain stem cell-like potential and GBM characteristics has provided us with a valuable model of the human disease for the present work.

The Notch signaling pathway is evolutionarily conserved and has a diverse impact on several cellular pathways and functions depending on the cellular context, the activating ligand as well as intervention from additional signaling pathways. The involvement of Notch in cell-fate decisions during development of the nervous system was originally outlined through studies in *D. melanogaster*, and it has been demonstrated that Notch signaling influences the balance between the NSC pool and its differentiated progeny through both lateral inhibition as well as inductive and restrictive cell fate determination.[18-25] The Notch receptors have been associated with cells in neurogenic areas such as the subventricular zone, the dentate gyrus, and the rostromigratory stream of the postnatal mammalian brain,[26-28] all areas where NSC are abundant.[29-31] Clinically, GBM are often located in close association to such areas,[4,32] thus it is no surprise that Notch pathway components often are found aberrantly expressed in GBM and thereof derived neurosphere cultures.[16,33-38] Furthermore, it has been suggested that Notch activity plays a central role in bCSC survival, tumorigenicity, and resistance mechanisms.[36,39-44]

In the present study, we seek to further dissect the relevance of Notch signaling in bCSC by investigating the importance of an active Notch pathway in GBM neurosphere cultures. We show that human-derived GBM xenograft cells cultured during NSC conditions in vitro contain cells with NSC-like characteristics. We further found that Notch pathway activation differed between the cultures investigated and that inhibition of Notch signaling, using the γ-secretase inhibitor (GSI) DAPT, which inhibits the activating cleavage of the Notch receptor,[44] hampered cell growth and primary sphere formation only in GBM neurosphere cultures with high endogenous Notch activity. However, endogenous Notch activity did not seem to be important for additional stem cell characteristics such as self-renewal and differentiation status. On the contrary, by introducing a constitutively active form of the Notch-1 receptor (ICN-1) and thereby exogenously activate the pathway, both cell growth and stem cell characteristics could be affected in all cultures. Taken together these results suggest that Notch signaling is important for the growth of GBM neurosphere cells with high endogenous Notch expression and activation and that Notch activity can be increased to a level where it influences the NSC-like characteristics of all cultures. Thus, Notch-directed GBM therapy for targeting the bCSC population is likely a promising alternative or supplement to existing treatment regimes.

Results

GBM neurosphere cultures harbor neural stem-like cells. In the past decade several groups have identified and characterized bCSC from GBM based on their ability to form neurospheres during stem cell culturing conditions, their self-renewing capacity based on sub-sphere formation, their multi-differentiation potential and the expression of NSC markers.[10,11,14] Here GBM neurosphere cultures were established from human-derived GBM xenograft tissue as previously described.[17] All cultures observed were able to form both primary spheres and spheres after dissociation, i.e., sub-spheres (**Fig. 1A**). To test the self-renewing capacity of the sphere-forming cells, sub-sphere formation was quantified as the number of spheres formed per 100 cells plated to approximately 35–45 spheres (**Fig. 1B**). It should be noticed

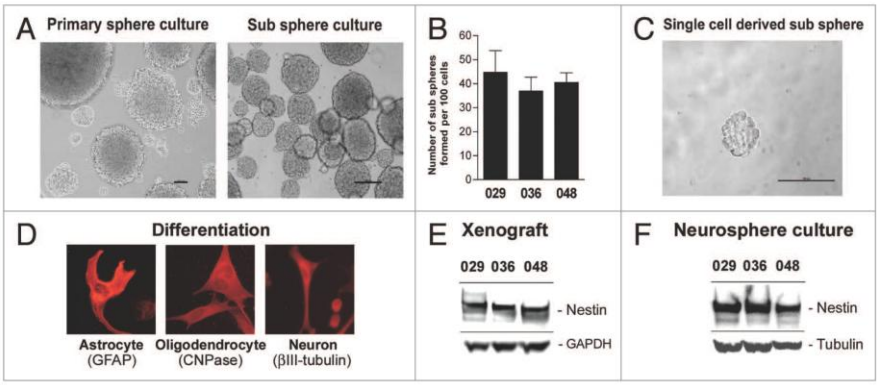

Figure 1. Neural stem-like cells are present in GBM xenografts. (**A**) Representative pictures of a primary and a sub sphere culture. Scale bar shows 100 μM. (**B**) Sub sphere formation in later cultures. Bar chart represents the mean number of sub spheres formed per 100 cells plated ± SEM. (**C**) Representative sphere formed from a single cell. (**D**) Representative pictures of differentiated sphere cells expressing GFAP (astrocytic marker), CNPase (oligodendrocytic marker), or βIII-tubulin (neuronal marker) upon serum addition. Expression was detected by immunocytochemical staining. WB analysis of the NSC marker Nestin in (**E**) subcutaneous GBM xenografts and (**F**) thereof derived neurosphere cultures.

that the sub-sphere forming potential varied between passages, in most cases increasing with increasing passage number (**Fig. S1**), indicating an enrichment of sphere forming cells in the culture. All cultures were, furthermore, able to form spheres from a single cell plated (**Fig. 1C**). Together these data indicate that the sphere-forming cells are self-renewing. To test whether multipotent cells were present in the cultures, dissociated sphere cells were subjected to serum-containing media in order to induce differentiation. Addition of serum made the cells grow adherently with a more differentiated morphology (data not shown). Immunocytochemical detection of GFAP, βIII-tubulin, and CNPase demonstrated that all cultures contained cells expressing neuronal and/or glial markers upon differentiation (**Fig. 1D**), suggesting that the cultures harbored cells with multi-differentiating potential. Finally, all three xenografts and thereof derived following passages of neurosphere cultures expressed high levels of the stem cell marker Nestin (**Fig. 1E and F**), compared with the commercial U251 GBM cell line used as a positive control[45] (data not shown). All cultures have been injected intracranially into SCID mice and grown as orthotopic tumors (unpublished data), and similar cultures have been re-implanted and successfully established as secondary subcutaneous tumors on the flanks of nude mice,[17] together suggesting that cells within the neurosphere cultures have maintained their tumorigenic potential. Based on these results we believe that the neurosphere cultures used in this study can be categorized as harboring a bCSC population.

GBM neurosphere cultures express varying levels of Notch-1 and the Notch transcriptional target Hes-1. The Notch receptors, their ligands and downstream mediators have been found aberrantly expressed in various grades and types of gliomas, commercial glioma cells lines as well as in glioma-derived neurosphere cultures and it has been demonstrated that the Notch pathway plays a role in glioma cell survival and maintenance.[16,33,39,40] We therefore wished to determine the Notch-1 protein level and the activity of the Notch pathway, as assessed by the Notch transcriptional target Hes-1, in the GBM neurosphere cultures and xenograft tumors used in this study. As shown in **Figure 2A** subcutaneous xenograft tumors from which the in vitro cultures were established expressed Notch-1 at various levels and the expression of Notch-1 was higher in the 029 and 036 xenografts than in the 048 xenograft. This expression pattern was also evident in the corresponding neurosphere cultures (**Fig. 2B**). Expression of the Notch target Hes-1 was likewise higher in the 029 and 036 xenografts and in vitro cultures, as compared with 048 (**Fig. 2A and B**). Taken together, the Notch-1 expression and pathway activation seems to be maintained from the xenografts to the in vitro cultures. Furthermore, we classified the 029 and 036 xenografts and cultures as having high endogenous Notch-1 expression and Notch pathway activation as compared with the 048 xenograft and culture which we classified as having low expression and activation.

Notch inhibition induces cell cycle arrest without cell death in cultures with high endogenous Notch expression and activity. To investigate the effect of Notch pathway inhibition on our in vitro cultures, dissociated neurosphere cells were treated with 5 μM DAPT for 2 weeks, where after the cells were harvested

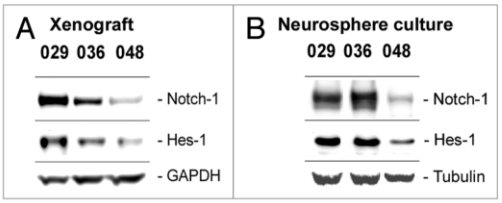

Figure 2. Expression of Notch-1 and the Notch target Hes-1 is maintained from xenograft to culture. Basal protein expression of the Notch-1 receptor and the Notch transcriptional target Hes-1 in (**A**) subcutaneous xenograft tumors from which the neurosphere cultures used in this study were derived and in (**B**) the xenograft-derived GBM neurosphere cultures.

for analyses. Q-RT-PCR analysis showed that the Hes-1 mRNA level was reduced in the 029 and 036 cultures upon DAPT treatment as compared with control (**Fig. 3A**). However, no effect was observed in the 048 culture. On protein level downregulation of Hes-1 was observed in the 029 and 036 cultures both after 2 weeks (**Fig. 3B**) and 3 d (**Fig. 3D**), whereas the downregulation was not as evident in the 048 culture, verifying the results from the Q-RT-PCR analyses. Thus, it is possible to hamper Notch signaling in cultures with high endogenous Notch-1 expression and pathway activation as assessed by Hes-1 expression, whereas Notch signaling in cultures with low Notch activity is less affected by γ-secretase inhibition.

To explore the effect of Notch inhibition on a functional level, cell cycle analysis was performed. Dissociated neurosphere cells were treated with 5 μM DAPT for three days and subsequently fixed and labeled with propidium iodide for flowcytometric quantification of DNA content. Upon analysis of the cell cycle, we found the G_0/G_1, non-dividing cell fraction to be elevated by approximately 10% in the DAPT treated 029 and 036 cells, whereas there was no consistent response in the 048 cells as evident by the large error bars (**Fig. 3C**). We were concerned that the increase in the 029 and 036 G_0/G_1 population reflected a decrease in the sub-G_0/G_1, dead cell fraction. However, when quantifying the fraction of dead cells in these cultures using trypan blue staining the total number of dead cells was not decreased upon treatment with neither 5 nor 10 μM DAPT (data not shown and **Fig. S2**). To further explore this, we analyzed the expression level of different apoptotic- and cell cycle markers upon treatment with 5 μM DAPT for three days. As shown in **Figure 3D** no change in the apoptotic markers cleaved caspase-3 or BAX could be observed upon Notch inhibition. The cell cycle regulator CDK4 was likewise not affected while a slight upregulation of the cell cycle inhibitor p21 could be detected in the 036 culture which correlates with inhibition of the cell cycle.

Tumorigenicity is obstructed by Notch inhibition in cultures with high Notch activation. We then tested if Notch inhibition targeted additional in vitro tumorigenic features of the GBM neurosphere cells such as the ability to grow independently of anchorage and cell–cell contact. As such, cells left untreated (DAPT-naïve) or cells pretreated with DAPT or DMSO were

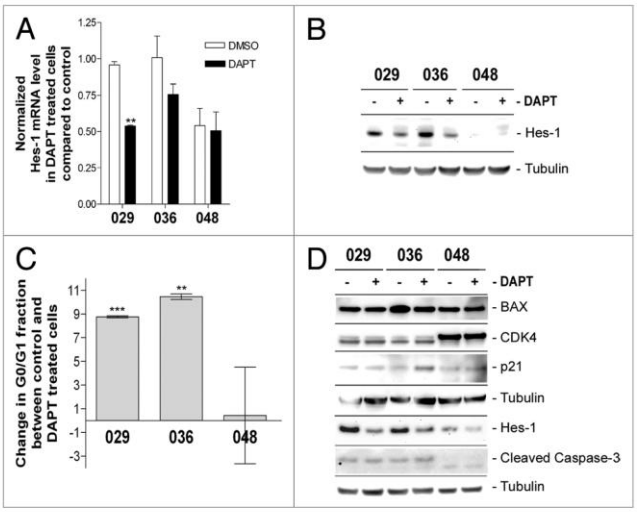

Figure 3. Notch inhibition more profoundly affects cultures with high Notch expression and activation. (**A**) Q-RT-PCR analysis of Hes-1 mRNA expression. Bars represent mean normalized Hes-1 mRNA expression ± SEM in control and DAPT treated cultures from two independent experiments performed in duplicates. Comparisons of means between control and treated were based on the unpaired t-test performed on the normalized data. Stars represent the difference between the mean of control and treated. $^{**}P < 0.01$. (**B**) WB detection of Hes-1 protein in DAPT and DMSO treated cultures. In (**A and B**) the cells were treated with 5 μM DAPT or DMSO for 2 weeks. (**C**) Cell cycle analysis of neurosphere cultures treated with 5 μM DAPT or DMSO for 3 days. Bars represent the difference in the G_0/G_1 fraction ± SEM between the DMSO control and the DAPT treated samples from three independent experiments. Positive bars correspond to an increase in the G_0/G_1 fraction in the DAPT treated samples compared with the control. Comparisons of means between control and treatment were based on the paired t-test performed on the untransformed data. Stars represent the difference between the mean of control and treated. $^{**}P < 0.01$, $^{***}P < 0.001$. (**D**) WB detection of Hes-1, cell cycle regulators (CDK4 and p21), and apoptotic markers (cleaved caspase-3 and BAX) in neurosphere cells treated with 5 μM DAPT or DMSO for 3 d.

plated in semi-solid agar with the addition of DAPT or DMSO. The number of clonogenic colonies formed was evaluated after two weeks. As displayed in **Figure 4A**, no effect on the colony formation was seen when DAPT-naïve cells were subjected to this assay. However, pretreatment with DAPT almost abolished the ability to form colonies in the 029 and 036 cultures (**Fig. 4B**), suggesting that these cells had lost some of their malignant phenotype. Again, no effect and thus no decrease in colony formation was observed in the low Notch expressing 048 culture. Representative pictures of a DAPT responsive culture are shown in **Figure 4C**.

DAPT treatment hampers neurosphere formation in primary culture but not in later passages and does not affect differentiation level. When GBM cells from tumor tissue are initially plated in NB-media in order to establish a primary in vitro culture, the bCSC can be identified by their ability to form neurospheres which are assumed to be clonal or aggregated proliferating cell aggregates.[47,48] Thus the fraction of GBM cells forming spheres in the primary culture can be interpreted as a semi-quantification of the stem-like cell population in the tumor. To study the importance of Notch signaling for the primary neurosphere growth and thus quantifying the size of the bCSC fraction, single cells from acutely dissociated subcutaneous xenograft GBM tissue were subjected to a primary sphere forming assay with the addition of different concentrations of DAPT. In this assay, we included additional subcutaneous xenograft tumors derived from two other GBM patients, 017p4 and 047p2, with low and high Notch-1 expression, respectively (**Fig. 5B**). The relative fraction of spheres formed per total number of cells plated is presented in **Figure 5A**. The ability to form spheres in the primary culture was notably inhibited in a DAPT concentration-dependent manner in the 029p5, 036p8, 036p15, and 047p2 cultures whereas no effect was observed in the 017p4 and 048p7 cultures. In **Figure 5B**, the Notch-1 expression in the xenograft tumors, from which the cultures used in the primary sphere assay were established, is presented. Together the results in **Figure 5A and B** indicate that DAPT treatment only affects primary sphere formation in cultures derived from xenografts defined as having high Notch-1 expression.

Others have shown that Notch pathway blockade reduces the self-renewal capacity in both normal NSC and in GBM sphere cultures as demonstrated by reduced sub-sphere formation upon γ-secretase inhibitor (GSI) treatment.[41,49] In order to investigate if Notch activity was important for the self-renewing capacity in our GBM neurosphere cultures, neurosphere cells were subjected to a sub-sphere assay with the addition of DAPT. The assay was performed both on DAPT-naïve and pretreated cells as with the soft-agar assay. As shown in **Figure 5C and D** no inhibitory effect of DAPT on sub-sphere formation in either of the setups was observed.

Stem-like cells have the capacity to uphold an immature state and produce more differentiated daughter cells,[47,50,51] and it is suggested that Notch expressing cells during normal development are prevented from undergoing differentiation.[52] To test if Notch activity plays a role in maintaining an undifferentiated pool of cells within our GBM neurosphere cultures, naïve or pretreated neurosphere cells, in parallel to the sub-sphere assay were harvested for WB analyses of markers representing the three neural lineages; GFAP for astrocytes, CNPase for oligodendrocytes, and βIII-tubulin for neurons. As shown in **Figure 5E and F**, no consistent change in the expression of these differentiation markers was detected in either the naïve or pretreated GBM neurosphere cells. Furthermore, no effect on the expression of the NSC marker Nestin was observed (**Fig. S3**).

Notch activation affects neurosphere stem cell characteristics and increases tumorigenicity. In order to study the effect of Notch pathway activation, we introduced a truncated, constitutively active form of the Notch-1 receptor (ICN-1) into our GBM neurosphere cultures by transient transfection. A time profile of total Notch-1 expression in ICN-1 and mock transfected neurosphere cells is shown in **Figure 6A**. Four hours after transfection a double band could be detected, where the upper band most likely represents the uncleaved transmembrane domain of endogenous Notch-1 and the lower band the intracellular Notch-1 domain, indicating successful transfection. At 20 and 40 h, the expression was further elevated compared with the mock transfected cells. It should be noticed that the transfection efficiency in general was highest in the 048 culture (~75%) and lowest in the 029 culture (<25%) and that it subsided after approximately 9 d, estimated as the fraction of green fluorescent cells (data not shown). When transfected cells were subjected to cell cycle analysis a decrease in the fraction of cells in the G_0/G_1 phase could be detected in all three cultures (**Fig. 6B**). The 048 culture displayed the largest decrease in the G_0/G_1 fraction, while the 029 culture did not respond to the same degree. Again, we were concerned that the decrease in the G_0/G_1 fraction reflected an increase in the sub-G_0/G_1 fraction. Therefore, ICN-1 and mock transfected cells were subjected to a soft-agar assay and the number of colonies was scored after 2 weeks. In general the ICN-1 transfected cells produced larger colonies than the mock transfected cells, indicating an increased cell cycle (representative photos are shown in **Fig. 6C**). When quantified, an increase in the colony forming potential was observed in the ICN-1 transfected cells as compared with the mock transfected cells (**Fig. 6D**). This effect was more pronounced in the low Notch-1 expressing 048 culture. Finally, as DAPT treatment did not affect the stem cell characteristics of the neurosphere cells as exemplified by lack of effect on sub-sphere formation and differentiation status (**Fig. 5B and C**), we performed the same experiments on ICN-1 transfected cells to explore if these stem cell characteristics could be affected by increasing the Notch activity. As demonstrated in **Figure 6E**, a slight increase in the number of sub-spheres formed was observed upon ICN-1 transfection. When analyzing the expression of neuronal and glial markers after ICN-1 transfection (**Fig. 6F**) all three markers were downregulated in the 029 culture implying de-differentiation, while the 036 and 048 cultures only showed downregulation of one of the markers, βIII-tubulin and GFAP respectively. The modest effect on sub-sphere formation and differentiation level might be due to the fact that the analyses were performed on the whole culture and not only the ICN-1 transfected cells. However, the above outlined results indicate that it is possible to activate Notch signaling to a degree where it affects the stem cell characteristics of the GBM neurosphere cultures.

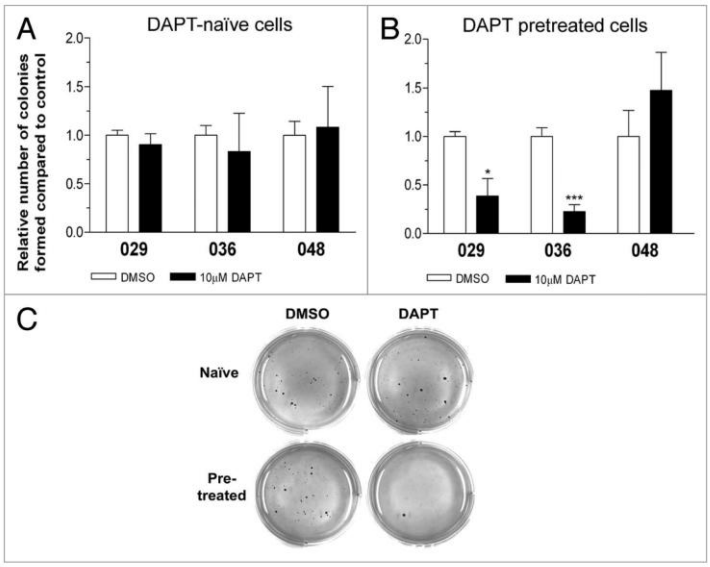

Figure 4. Notch inhibition hampers in vitro tumorigenic potential. Colony formation assay in soft-agar with the addition of 10 μM DAPT or DMSO performed on (**A**) DAPT-naïve cells or (**B**) DAPT-pretreated cells. Bars shows the relative mean of colonies formed after 14 d ± SEM. Stars represent the difference between the mean of control and treated. *$P < 0.05$, ***$P < 0.001$. (**C**) Representative photos of the colony formation assay.

Discussion

Given the potential role for bCSC in tumor development, maintenance, resistance to therapy, and relapse, it is of great importance to further understand the mechanism(s) involved in bCSC regulation. In the present study we have investigated the role of Notch signaling for the stem cell-like and tumorigenic potential of GBM neurosphere cells. We have established GBM neurosphere cultures from human-derived GBM xenografts and identified these as harboring cells with bCSC features. All cultures investigated in this study expressed Notch-1 and the downstream target Hes-1 at various levels. In cultures with high Notch-1 and Hes-1 expression, inhibition of Notch signaling led to decreased cell growth as displayed by decreased number of clonogenic colonies, most likely as a consequence of cell cycle inhibition. In opposite, when introducing a constitutively activated variant of Notch-1, an increase in cell growth was obtained. Furthermore, both the self-renewing capacity and de-differentiation level were increased in all cultures upon Notch activation. These stem-like

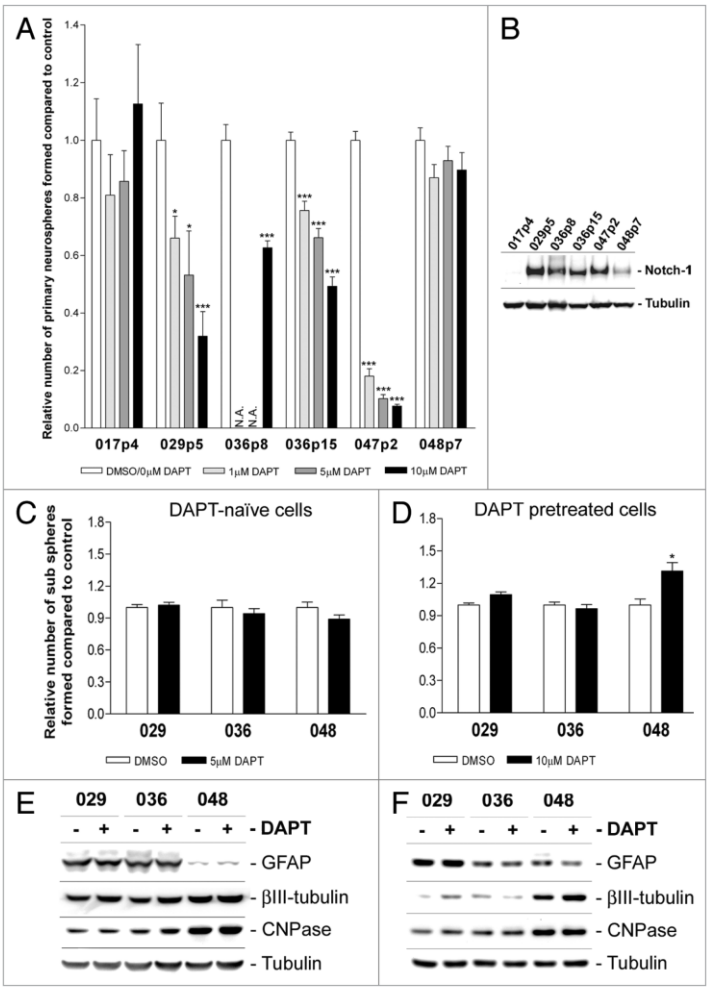

Figure 5. DAPT treatment affects primary neurosphere formation but not sub sphere formation or differentiation level. (**A**) Primary sphere formation assay performed on acutely dissociated GBM xenograft cells with the addition of 0, 1, 5, or 10 μM DAPT. Notice that 0 μM DAPT was used as control for 029p5, 036p8, 047p2, and 048p7, while DMSO was used for 017p4 and 036p15. (**B**) WB detection of Notch-1 in xenograft tumor tissue from which the acutely dissociated GBM cells for the primary sphere assay were derived. (**C**) Sub-sphere formation assay performed on naïve cells treated with 5 μM DAPT or DMSO or (**D**) pretreated cells with the addition of 10 μM DAPT or DMSO. In (**A, C, and D**) bars show relative mean of spheres formed after 14 d ± SEM. Stars represent the difference between the mean of control and treated. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. N.A., not analyzed. (**E and F**) WB detection of markers for differentiation in neurosphere cells treated in parallel to (**C and D**) respectively. GFAP for astrocytes, βIII-tubulin for neurons, and CNPase for Oligodendrocytes. Naïve cells are displayed in (**E**) and pretreated in (**F**).

features were, however, not affected by inhibition of endogenous Notch signaling. Nevertheless, as the primary sphere forming capacity was clearly reduced upon Notch inhibition, our data together imply that active Notch signaling plays a role in maintaining the bCSC population in the high Notch-1 expressing GBM cultures.

Others have shown that the Notch receptors and components of the Notch signaling pathway are differently expressed in GBM as well as in GBM-derived cultures.[16,33-37] In consensus, two of the cultures used in this study, the 029 and 036 cultures, showed elevated expression of the Notch-1 receptor and Hes-1 as compared with the 048 culture, indicating that the Notch pathway has a higher activation level in the former cultures. It should be considered that additional Notch receptors (Notch-2–4) and downstream targets, other than Hes-1, could be of importance for the outcome of Notch activity in these cultures and we can therefore not exclude that 048 has high Notch pathway activity. However, as this culture in general displayed insensibility toward Notch inhibition, this is not a likely scenario. Substantiating this, Hes-1 expression was most prominently downregulated upon DAPT treatment, as has been shown by others,[40,41] in cultures that were also affected on a functional level, namely the 029 and 036 cultures. Hes-1 is a member of the bHLH family and mediates Notch signaling by acting as a transcriptional repressor and thousands of potential binding sites for Hes-1 have been identified.[53] Thus attenuation of Hes-1 expression has multiple potential functional effects. It is moreover important to hold in mind that several additional Notch targets have been reported. Besides Hes-2–7, also the bHLH transcriptional repressors Hey-1, -2, and -L,[54] GFAP[55] that, apart from being an astrocytic marker, also is expressed in postnatal NSC,[56,57] the NSC marker Nestin[58,59] as well as the cell cycle regulators p21 and Cyclin D1[46,60,61] have been suggested as Notch targets and the list continues to elongate. Thus, as active Notch signaling has multiple targets and each target affects even more downstream mediators, it is naïve to conclude that Hes-1 is the sole explanation for the effects observed upon Notch modulation. Nevertheless, the functional effect of Hes-1 seems to be more prominent and severe than the effect of the other Hes proteins

(reviewed in ref. 62). Furthermore, the data presented here suggest that the effect of γ-secretase inhibition can be predicted by the Hes-1 expression level, as DAPT treatment only had a functional effect on GBM neurosphere cultures with high endogenous Hes-1 expression. It could therefore be speculated that Hes-1 could either be used as a predictive marker for Notch-directed targeted therapy or function as a therapeutic target itself.

To explore the functional role of Notch in our neurosphere cultures, different cellular in vitro assays evaluating cell growth were utilized. These assays combined can be interpreted as a measurement of the in vitro tumorigenic potential of the cells examined. Notch inhibition resulted in an increase in the G_0/G_1 cell fraction of the 029 and 036 cultures, whereas 048 remained unaffected. Furthermore, no obvious change in the number of dead cells was observed in 029 and 036, and an increase of p21 was seen in 036, a phenomenon also described by others.[46] These data indicate that inhibition of Notch signaling in GBM neurosphere cells with high endogenous Notch activity leads to cell cycle arrest or slowing of the cell cycle without induction of cell death, a notion supported by our results showing progression through G_0/G_1 upon artificial Notch activation. Others have shown that Notch-1 knock down leads to upregulation of p21 together with cell cycle arrest in G_0/G_1.[46,61] These studies were however performed in different cancer types and they moreover detected an increase in apoptosis which is in contrast to our findings. Nevertheless, our results support previously published data on the effect of Notch inhibition on the cell cycle in NSC and various cancer types including GBM[40,46,60,61,63,64] and as such we suggest that Notch activity is involved in GBM neurosphere cell growth and perhaps more specifically in the G_0/G_1–S phase transition. This statement is further supported by our results from the soft-agar clonogenic growth assay, which is a commonly used in vitro tumorigenic assay as it evaluates the capability for anchorage- and cell–cell contact independent clonogenic growth, a capability only cancer cells posses.[65,66] We did not observe any difference in colony formation when naïve cells were treated with DAPT. However, when the cells were pretreated and then subjected to the assay the clonogenic growth potential was clearly reduced in the 029 and 036 cultures, which is in line with previous data from Fan et al.[40] This discrepancy between naïve and pretreated cells might reflect that Notch inhibition

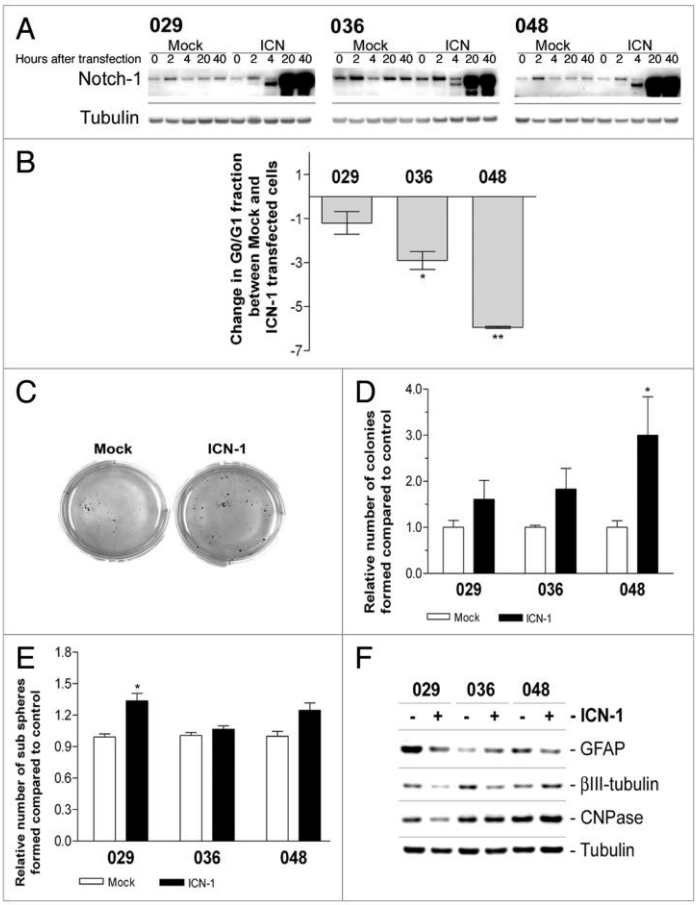

Figure 6. Transfection with ICN-1 results in opposite effect from DAPT treatment and affects stem cell characteristics. (**A**) Expression of total Notch-1 in cultures transfected with ICN-1 or Mock plasmid after 0, 2, 4, 20, or 40 h. (**B**) Cell cycle analysis of neurosphere cultures transfected with ICN-1 or Mock and left for 3 d. Bars represent the difference in the G_0/G_1 fraction ± SEM between Mock and ICN-1 transfected samples from at least two independent experiments performed in mono- or duplicates. Negative bars correspond to a decrease in the G_0/G_1 fraction in the ICN-1 transfected samples compared with the mock samples. Comparisons of means between Mock and ICN-1 were based in the paired t-test performed on the untransformed data. (**C**) Representative photos of soft-agar assay performed on cells transfected with ICN-1 or Mock. (**D**) Quantification of colonies formed in the soft-agar assay with ICN-1 or Mock transfection. Bars shows the relative mean of colonies formed after 14 d ± SEM. (**E**) Sub sphere formation assay performed on cells transfected with ICN-1 or Mock. Bars show relative mean of spheres formed after 14 d ± SEM. For (**B, D, and E**) stars represent the difference between the mean of control and treated. $*P < 0.05$, $**P < 0.01$. (**F**) WB detection of markers for differentiation in neurosphere cells treated parallel to (**E**). GFAP for astrocytes, βIII-tubulin for neurons, and CNPase for oligodendrocytes.

leads to increased differentiation of neurosphere forming cells, as has been shown by others in NSC, glioma cells and bCSC,[34,41,52] into lineage restricted progenitor cells that are still able to proliferate and form colonies. Progenitor cells do, however, not have the capability to self-renew and become proliferative exhausted over time (reviewed in refs. 67 and 68) and therefore DAPT-pretreated neurosphere forming cells that had have differentiated into progenitor cells would not form colonies upon replating. On the opposite, when the cells were transfected with the constitutively activated Notch-1 receptor we found an elevated capability to form colonies in soft agar. The effect of Notch activation was observed in all 3 cultures. However, as with the cell cycle analysis it was more prominent in 048, most likely due to the high transfection efficiency in this culture as compared with the other cultures. It could also be speculated that cells with high endogenous Notch activation, like the 029 and 036 cultures, do not benefit from increased ICN-1 levels to the same degree as the 048 culture with low Notch activation. Nevertheless, the above discussed results suggest that activated Notch signaling leads to increased clonogenic proliferation and thus enhanced malignant potential.

The above data support results recently published by Fan et al., who showed that Notch inhibition using different γ-secretase inhibitors led to decreased cell viability and Hes-1 expression in GBM neurosphere cultures whereas transfection of the cells with the intracellular Notch-2 receptor resulted in increased cell growth.[40] They stated that Notch-2 activation was sufficient to promote GBM neurosphere cell growth. It is however important to point out that γ-secretase inhibitors do not specifically target one Notch receptor, but instead inhibit activation of all 4 receptors. As such, it is at this point not possible to conclude if the two receptors are equally important for GBM neurosphere cell growth or if there is some kind of redundancy between the two receptors with regard to their functional relevance.

The role of Notch signaling during normal development including NSC regulation is firmly established; however, we are only beginning to elucidate its role in bCSC regulation.[36,40-42] NSC and bCSC are commonly identified by their sphere forming capacity in culture, and thus the number of spheres formed in primary culture from acutely dissociated tumor tissue can be interpreted as a semi-quantification of the bCSC population in the tumor tissue sample. A correlation between primary neurosphere formation and survival of glioma patients has been suggested[69,70] and it has been shown that the vast majority of GBM tumors could be established and maintained as neurosphere cultures, whereas lower grade gliomas mostly lacked this ability.[70] We have successfully established neurosphere cultures from every human-derived GBM xenograft tumor attempted in this and previous studies.[17] Furthermore, here we show that the ability for primary neurosphere formation could be reduced in a DAPT concentration-dependent manner in cultures with high endogenous Notch activity, indicating a decline in the bCSC population in the primary culture upon Notch inhibition. These observations are in line with previously published results showing that GSI treatment decreases the number of primary spheres formed from GBM and normal NSC[41] and support the idea of Notch inhibition as a way to target the bCSC population and improve patient survival. One might question, that despite the low level of Notch-1 protein in the 017p4 xenograft, these cells were still able to form spheres in the primary culture. This observation does, however, merely imply that additional pathways play a role for the bCSC capacity in the sphere forming GBM cells with low Notch activity.

Surprisingly, when the sphere assay was performed on latter passage cultures, no effect of DAPT treatment on neurosphere formation was observed. Thus, it seems as if Notch activity is only important for maintenance of the bCSC in the primary culture and not in later passages. This hypothesis was supported=when we found no or little effect of DAPT on the expression of differentiation markers, which suggests that the differentiation level of the cultures was maintained despite of Notch inhibition. Taken together, these data suggest that although it is possible to inhibit neurosphere growth in primary cell cultures, neither the self-renewing capacity nor differentiation level, both reflecting stem-like features, are dependent on Notch signaling in any of our long-term neurosphere cultures, although Notch-1 expression and activity, as assessed by Hes-1 expression, are maintained.

One might argue that the primary, sub-sphere, and soft-agar assays all reflect a stem cell feature, namely the capability to form proliferating cell aggregates, and Notch inhibition as such should result in similar outcome in the different assays. There are, however, fundamental differences between the three assays. First of all, the soft-agar assay differs from the sub-sphere assay, as the cells are embedded in a semi-solid agar with no possibility of cell–cell contact and signaling and are thus dependent on growth factors in the media and endogenic signaling for proliferative activity. In contrast, in the sub-sphere assay the cells float around in the media and are able to interact and as such, the formation of a neurosphere is not by certainty the result of clonogenic growth in the sub-sphere assay, which is in contrast to the case in the soft-agar assay. Furthermore, the cell–cell interaction in the sub-sphere assay might enable activation of additional pathways that overrule the Notch inhibition. Indeed pathways, such as the EGFR, Wnt, and SHH pathways, have been shown to be important for bCSC maintenance and growth[71,72] (and reviewed in ref. 73), and it has furthermore been demonstrated that aggregation promotes proliferation of NSC.[48] This functional redundancy of pathways might reflect the fact that normal stem cells are crucial for upholding the organism and thus have multiple possibilities for self maintenance and protection. Second, when acutely dissociated cells from the xenograft tumor biopsy initially were subjected to NB-media, no cell sorting was performed. As such, all the different cell types present in the tumor were most likely present during establishment of the primary neurosphere culture. Over time and with increasing passages, the NB-media would select for cells with stem cell-like potential and their derivates, and fully differentiated cells that were present in the original tumor, such as stromal and endothelial cells would be eliminated. As activation of Notch signaling requires receptor–ligand interaction and as Notch signaling is known to occur between two adjacent cells (although cell autonomous ligand-receptor interaction has been demonstrated[74]), it is reasonable to speculate that the Notch signaling is activated by different means in

the primary culture as compared with later passage cultures. In that context, it has been shown that the Notch-1 receptor and the Notch ligands Dll-1 and -3 and Jag-1 and -2 are expressed in different brain regions[26] and thus most likely by different cell types and that Dll-4 is expressed by GBM endothelial cells.[75] This means that in the primary sphere assay, Notch signaling in the bCSC could be activated by ligand-bearing cells of different origin, while it in the later passage cultures occurs between cells of the same origin. As such, it is possible that these two scenarios lead to different Notch activation levels in the primary and later passages cultures, and also that there is a threshold level for Notch activity, over which it has an influence on the stem cell-like features of the bCSC. Indeed, it has been shown that modulation of Notch activity has different outcome depending on the endogenous Notch activation level in NSC.[76] One could thus argue that in the primary culture Notch activity would be activated over the threshold level and thereby able to exert its effect on the bCSC population, whereas the activity would be below the threshold level in the later passage culture, and thus inhibition of Notch signaling would have no or little effect on the stem cell characteristics. This hypothesis was substantiated by our results using artificially activated Notch signaling. Here we did obtain an effect on the sub-sphere forming potential and differentiation level. The effect was not as convincing as the effect on the cell growth potential, but there was nevertheless a tendency toward an increase in the number of sub-spheres and a decrease in the expression of differentiation markers suggesting that the bCSC population was expanded. This was further supported by an increase in the clonogenic growth potential observed in the soft-agar assay when using constitutively activated Notch. Thus, even if inhibition of endogenous Notch signaling did not affect the stem cell characteristics of later passage neurosphere cultures, the Notch activity could be artificially boosted to a level where it indeed affected the stem cell-like potential.

Another explanation for the lack of effect from DAPT treatment on the stem-cell features in later passages could be that the bCSC population displaying Notch activity might only constitute a small fraction of the entire culture. It is thus not unlikely that the effect from Notch inhibition on the bCSC characteristics is too subtle to be appropriately detected. However, as we detected a pronounced decrease in clonogenic cell growth possibly due to reduced cell cycling upon inhibition of endogenous Notch signaling, our results indicate that also tumor cells without stem cell-like features, representing a non-bCSC population, are affected by Notch inhibition as has been shown by others.[34,39]

In conclusion, we have shown that it is possible to target both the tumorigenic potential and the stem cell-like features of GBM neurosphere cells by Notch pathway modulation and that Notch inhibition only affects cells with high endogenous Notch activity. The differences in Notch expression and activation, leading to differences in sensitivity toward Notch inhibition might reflect that the neurosphere cultures used in this study belong to different subtypes of GBM. Indeed, it has recently been published that GBM tumors can be divided into several subclasses based on their molecular expression profile and clinical appearance.[35,38,77] As such the 029 and 036 cultures might belong to a subclass characterized by Notch activation, whereas the 048 culture could belong to a subtype in which Notch signaling is of less importance. Taken together we suggest that using a treatment regime that includes Notch inhibition together with stratifying patients according to Notch pathway activity using, e.g., subtype profiling, could improve the prognosis for GBM patients by reducing both the bCSC and the non-bCSC populations.

Materials and Methods

Ethics statement. In a previous study[17] patient GBM material (GBM_CPH017, GBM_CPH029, GBM_CPH036, GBM_CPH047, and GBM_CPH048) was obtained from surgery at Copenhagen University Hospital, Denmark, approved by the Scientific Ethical Committee for Copenhagen and Frederiksberg: (KF) 01-034/04 with the patients' informed consent and maintained over several passages as subcutaneous xenografts on the flank of nude mice, according to Danish legislation.

Establishing and maintaining GBM xenografts as in vitro neurosphere cultures. Neurosphere cultures were established from acutely dissociated patient-derived subcutaneous xenograft tumors and maintained in NB-media (Neurobasal-A media supplemented with 1× B-27 supplement, 1× L-glutamine, 10 ng/ml basic fibroblastic growth factor (bFGF), 10 ng/ml epidermal growth factor (EGF), 1% Pen Strep (penicillin–streptomycin) (all from Invitrogen), and 10 ng/ml leukemia inhibitory factor (LIF) (Chemicon) as previously described.[17] The cells were cultivated in uncoated cell culture flasks (NUNC) in a humidified chamber with 5% CO_2 at 37 °C. Fresh media was added twice a week and spheres were mechanically dissociated at every passage. Three different neurosphere cultures were used in the present study: GBM_CPH029p7, NGBM_CPH036p7, and NGBM_CPH048p6, unless otherwise stated, corresponding to three different patient-derived xenograft tumors. pX indicates the mouse passage from which the individual cultures were established. The prefix "N" refers to that the xenograft has been transplanted onto rats for a period to remove mouse hepatitis infection. For simplicity, the three cultures are designated 029, 036, and 048 respectively.

Modulation of Notch signaling in neurosphere cultures. For Notch inhibition studies, neurospheres were dissociated into single cells and plated in NB-media in either petri dishes (10 cm Ø, Nunc) for pretreatment or directly in a suitable assay plate (see below) and allowed to grow overnight (ON) unless otherwise stated. The latter will be designated DAPT-naïve cells. N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-butyl ester (DAPT, γ-secretase inhibitor [GSI] IX, Calbiochem) dissolved in dimethyl-sulfoxide (DMSO) hybri-max (Sigma) was added the following day, unless otherwise stated, at a concentration of either 1, 5, or 10 μM. Equal volumes of DMSO were used as control, unless stated otherwise. Pretreated cells were re-dissociated at day seven, plated in a suitable assay plate corresponding to the DAPT-naïve cells and re-treated with compounds equivalent to pre-treatment. The DAPT concentrations used in this study were based on previous studies using DAPT and additional γ-secretase inhibitors.[40,41,44,63]

For Notch activation studies, the following plasmid vectors were used: pcDNA3.1(+)-i.c.Notch-1 (kindly provided by J. Aster) coding for the constitutively activated intracellular Notch-1 domain (ICN-1) and pcDNA3.1(+) (Invitrogen) used as a control. Neurospheres were dissociated into single cells and transfected for 3 h with 3 μg plasmid using 12 μL Lipofectamine 2000 in Opti-MEM Reduced Serum Medium (both from Invitrogen). Transfection efficiency was estimated by co-transfection with pEGFP-N1 (Clontech) and manual scoring of the proportion of cells expressing EGFP by fluorescence microscopy. Following transfection, the cells were re-plated in NB-media and left for one day before subjected to an assay unless otherwise mentioned.

Differentiation and immunocytochemistry. Spheres were mechanically dissociated into single cells and plated in an 8-well chamber slide (NUNC) in 100 μl of NB-media at a density of 25–100 cells/μl. An amount of 300 μl Dulbecco's modified Eagle medium (DMEM), with 10% fetal calf serum (FCS) and 1% Pen Strep (all from Invitrogen) was added. The cells were left to differentiate for 7 d and subsequently fixed using a 1:1 mix of acetone and methanol, permeabilized in a Triton X-100 solution and blocked using 3% bovine serum albumin (BSA, Sigma). The cells were then incubated ON with primary antibody diluted in 1% BSA in PBS followed by tetramethyl rhodamine isothiocyanate (TRITC) conjugated secondary antibody. Object glasses were mounted with Glycergel (Dako). TRITC was excitated at 557 nm and positive cells were visualized using Nikon Eclipse TS100 microscope, Nikon digital camera DXM1200F, and software NIS-Elements F 3.0 Nikon. Primary antibodies: Rabbit polyclonal anti-Glial Fibrillary Acidic Protein (GFAP, Dako), Mouse monoclonal anti-CNPase [11-5B] (Abcam) and Mouse monoclonal anti-tubulin, β III isoform [TU-20] (βIII-tubulin, Chemicon). Secondary antibodies: TRITC-Conjugated polyclonal rabbit anti-mouse IgG, TRITC-Conjugated polyclonal swine anti-rabbit IgG (both from Dako).

Western blotting. Whole cell lysates were prepared from cell pellets by sonication in ice-cold RIPA buffer (50 mM Tris-HCL [pH 7.4], 1% NP40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with protease and phosphatase inhibitor mixture II and III (Calbiochem). Protein concentrations were determined using the BCA protein assay (Pierce) according to manufactures instructions. For western blotting (WB) 30–50 μg protein was separated on either 4–12% NuPAGE Bis-Tris gels or 3–8% NuPAGE Tris-Acetat gels (Invitrogen) and electroblotted onto nitrocellulose membranes (Invitrogen) according to manufacturers' protocol. The membranes were then blocked for 1 h in 5% non-fat milk at room temperature (RT) and incubated with primary antibody diluted in 5% non-fat milk ON at 4 °C followed by horseradish peroxidase (HRP) conjugated secondary antibodies for 1 h at RT. Blots were developed using the SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and the UVP, BioSpectrum AC Imaging System and VisionWorks LS software (UVP). Primary antibodies: goat polyclonal anti-Notch-1 [S-20], rabbit polyclonal anti-GAPDH (all from Santa Cruz), rabbit polyclonal anti-Hes-1 (kindly provided by Dr Tetsou Sudo, Toray Industries Inc.), mouse monoclonal anti-Nestin [10C2], mouse monoclonal anti-Tubulin, β III isoform [TU-20] (both from Chemicon), rabbit monoclonal anti-cleaved caspase-3 [5A1E], rabbit monoclonal anti-BAX, mouse monoclonal anti-CDK4 [DCS156], mouse monoclonal p21 Waf1/Cip1 [DCS60], rabbit monoclonal anti-α-Tubulin [11H10] (all from Cell Signaling), rabbit polyclonal anti-Glial Fibrillary Acidic Protein (GFAP, Dako), mouse monoclonal anti-CNPase [11–5B], (Abcam). Secondary antibodies: rabbit polyclonal anti-mouse IgG, rabbit polyclonal anti-goat IgG and swine polyclonal anti-rabbit IgG (all from Dako).

Quantitative real-time polymerase chain reaction. Total RNA was extracted from cell pellets using QIAshredder columns and RNeasy Mini Kit (both from Qiagen) according to manufacturer's protocol. All RNA was DNase treated using the RNase-Free DNase Set from Qiagen. cDNA was synthesized followed by RNase H treatment according to manufacturers protocol using SuperScript III Platinum Two Step q-RT-PCR kit with SYBR green (Invitrogen) which was also used for the subsequently quantitative real-time PCR (Q-RT-PCR) reactions. The ΔΔ-Ct method was used for calculating the normalized gene expression. All data was normalized to the expression of three housekeeping genes (TOP1, CYC1, and EIF4A2) included in the human geNorm housekeeping gene selection kit (Primerdesign). Primers used: Hes-1 forward: 5'-AGCGGGCGCA GATGAC-3', Hes-1 reverse: 5'-CGTTCATGCA CTCGCTGAA-3'.

Primary sphere assay. Single cells from acutely dissociated xenograft tissue were plated in 96-well plates at a density of 10 cells/μl in 200 μl NB-media and directly treated with 1, 5, or 10 μM DAPT. For control 0 μM DAPT or equal volumes of DMSO was added. At day 14, the number of spheres per well was scored and primary sphere frequency calculated. It should be mentioned that the cultures presented in the primary sphere assay are not derived from the same xenograft tumor passage as the cultures used in the additional in vitro experiments, although they are derived from the same original patient tumor. The cultures presented in the primary sphere assay are NGBM_CPH017p4, NGBM_CPH029p5, NGBM_CPH036p8, NGBM_CPH036p15, NGBM_CPH047p2, and NGBM_CPH048p7. For simplicity, the cultures are designated 017p4, 029p5, 036p8, 036p15, 047p2, and 048p7 respectively. 017p4 and 047p2 are xenograft tumors derived from two additional patient tumors and only used in this assay. Furthermore, the 017p4 tumor has been cultured in vitro in NB-media between the primary xenograft tumor and the xenograft tumor from which the primary sphere assay was performed. For the 029p5, 038p8, 047p2, and 048p7 cultures, 0 μM DAPT was used as a control. For the 017p4 and 036p15, DMSO was the control.

Sub-sphere assay. Dissociated neurosphere cells from later passages, either transfected with ICN-1, DAPT-naïve, or pretreated with 10 μM DAPT as described above, were plated in 96-well plates at 100 cells per well in 200 μl NB-media. Cells were treated with 0, 5 or 10 μM DAPT and the total number of spheres was scored after 2 weeks. In addition dissociated sphere cells were plated by limiting dilution at 0.5 cells/well without modulation for observation of single cell-derived spheres.

Cell cycle analysis. Dissociated sphere cells were either transfected with ICN-1 or treated with 5 μM DAPT for 72 h where after the cells were harvested, fixed in 70% ice cold ethanol and resuspended in a propidium iodide solution (50 μg/ml of propidium iodide, 10 mM Tris, 5 mM $MgCl_2$, 10 μg/ml of ribonuclease A, and 1 μl/ml of NP-40 (propidium iodide and ribonuclease A were purchased from Sigma-Aldrich). DNA content of the samples was quantified using a FACS Canto flow-cytometer and data were analyzed using the FACS Diva software program (BD Biosciences).

Soft-agar assay. 1×10^5 dissociated neurosphere cells, either transfected with ICN-1, DAPT-naïve, or pretreated with 10 μM DAPT as described above, were plated in semisolid agar with NB-media in a 6-well plate. DAPT-naïve and pretreated cells were supplemented with 10 μM DAPT in the assay. At day 14, the colonies were stained with 0.005% crystal violet and the number of colonies was visualized by the UVP BioSpectrum AC Imaging System (UVP) and manually quantified.

Statistics. Except for western blot analyses, data from in vitro cellular assays were obtained from at least 3 independent experiments each performed in duplicates or more unless otherwise stated. The primary sphere assay was, however, only performed once and in sextuplicates, as repeats were not possible. Data are shown as mean ± standard error of the mean (SEM) unless otherwise stated. For statistic analyses data was log-transformed and comparisons of means between control and treatment were based on the 1-way ANOVA test at 0.05 significance level unless otherwise stated.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cbt/article/24595.

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Supplemental Material to:

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Level of Notch activation determines the effect on growth and stem cell-like features in glioblastoma multiforme neurosphere cultures

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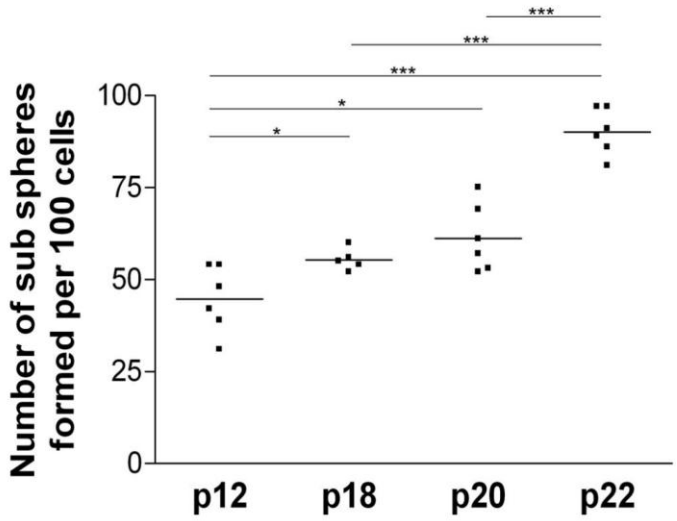
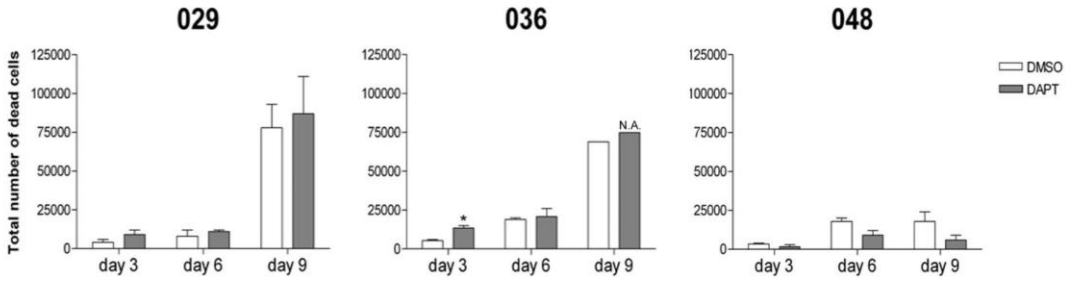
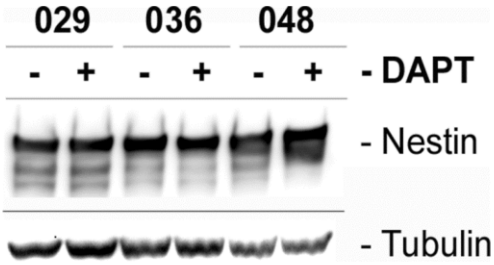


Supplementary Figure S1. Sub-sphere formation in different passages of the 029 culture. Sub sphere assay performed on different *in vitro* passages of the same neurosphere culture revealed a tendency to increased sub sphere formation with increasing passage number. The 029 culture is used as an example, but the trend was the same with the 036 and the 048 cultures. Dot-plot shows the number of spheres formed per 100 cells plated. Lines represent mean number of spheres. pX = *in vitro* passage number. Comparisons of means from one passage to another were based on the unpaired t-test performed on the untransformed data. Stars represent the difference between the mean of two passages. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.



Supplementary Figure S2. Dead cell count. For quantification of the proportion of dead cells, dissociated neurosphere cells were plated in NB-media, 3000 cells at 10 cells/µl, in a 6 well plate and treated with 10µM DAPT or DMSO. At day 3, 6 and 9, cells from duplicate wells were harvested, dissociated and stained with Trypan Blue Stain 0.4% (Invitrogen, Taastrup, Denmark). The proportion of dead cells was quantified as the number of Trypan Blue positive cells counted in a hemocytometer. The bar chart shows mean number of dead cells +/- SEM in neurosphere cultures treated with 10µM DAPT or DMSO. Comparisons of means between treated and control on a specific day were based on the unpaired t-test performed on the untransformed data. Stars represent the difference between the mean of control and treated. *: $p<0.05$. N.A. = not analyzed. No decrease in the total number of dead cells in the high Notch expressing 029 and 036 cultures treated with DAPT could be observed. Experiments with 5µM showed the same tendency (data not shown).



Supplementary Figure S3. Protein expression of Nestin in DAPT naïve neurosphere cells treated with 5µM DAPT. When neurosphere cells were dissociated and directly treated with 5µM DAPT and subsequent harvested for protein analysis by WB, no change in the expression of the NSC marker Nestin could be detected when compared to DMSO treated cells.

7. Results - Manuscript IV

Inhibition of Notch signaling alters the phenotype of orthotopic tumors formed from glioblastoma multiforme neurosphere cells but does not hamper intracranial tumor growth

Running title: Notch signature in brain cancer stem-like cells tumor formation

By:

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ABSTRACT

Background: Brain cancer stem-like cells (bCSC) are cancer cells with neural stem cell (NSC)-like properties found in the devastating brain tumor Glioblastoma Multiforme (GBM). bCSC are proposed a central role in tumor initiation, progression, treatment resistance and relapse and as such present a promising target in GBM research. The Notch signaling pathway is often deregulated in GBM and we have previously characterized GBM derived bCSC cultures based on their expression of the Notch-1 receptor and found that it could be used as predictive marker for the effect from Notch inhibition. The aim of the present project was therefore to further elucidate the significance of Notch activity for the tumorigenic properties of GBM derived bCSC.

Methods: Human derived GBM xenograft cells have previously been established as NSC-like neurosphere cultures. Notch inhibition was accomplished by exposing the cells to the γ-secretase inhibitor DAPT, prior to gene expression analysis and intracranial injection into immunocompromised mice.

Results: By analyzing the expression of several Notch pathway components, we found that the cultures indeed displayed different Notch signatures. Surprisingly, when DAPT treated cells were injected into the brain of immunocompromised mice, no increase in survival was obtained regardless of Notch pathway signature and Notch inhibition. We did however observe a decrease in the expression of the stem cell marker Nestin, an increase in the proliferative marker Ki-67 and an increased number of abnormal vessels in tumors formed from DAPT treated, high Notch-1 expressing cultures, when compared to the control.

Conclusion: Based on the presented results we propose that Notch inhibition partly induces differentiation of bCSC, and selects for a cell type that more strongly induces angiogenesis if the treatment is not sustained. However, this more differentiated cell type might prove to be more sensitive to conventional therapies.

INTRODUCTION

The brain tumor glioblastoma multiforme (GBM) is among the most lethal malignancies in adults and it affects 3.5/100,000 persons every year in western countries[1]. The survival probability is 57% the first year after diagnosis and recurrence is reported in 99% of patients within 7 years [2] why GBM thus fare is considered incurable. Brain cancer stem-like cells (bCSC) present a novel target in the search for improved anti-GBM therapy as they are suggested to play a pivotal role in tumor initiation, progression, treatment resistance and relapse[3-8]. The bCSC are characterized by their neural stem cell (NSC)-like features and tumorigenic properties, as they possess self-renewing and multipotent abilities as well as *in vivo* tumor forming potential[3, 9-11]. The search for regulators that might be of importance for maintenance of the bCSC population is thus considered a key quest in GBM research. The Notch signaling pathway is known to be important for maintenance of the normal NSC population during development as this pathway regulates the balance between the NSC pool and its differentiated progeny[12, 13], and it is believed that the outcome of Notch signaling in cancer reflects its role in the development of the corresponding normal tissue. In this context it has been demonstrated that Notch signaling is deregulated in GBM[14-16] and that bCSC are sensitive to Notch inhibition[16-18]. Elevated expression of Notch signaling pathway component has recently been associated with the classical GBM sub-type identified by global gene expression[19]. Sub-grouping of GBM tumors based on gene expression could potentially assist clinicians in the future when stratifying patients to the most optimal targeted treatment as has been the case for receptor tyrosine kinase HER2 and the estrogen receptor positive breast cancers[20-22]. Thus, by classifying GBM tumors into several sub-types it might be possible to identify new molecular targets, essential for maintenance of a specific sub-type. In line with this, we have previously shown that the level of Notch activity determines the effect of Notch inhibition in GBM neurosphere cultures *in vitro*, as growth and stem cell-like features only were affected in cultures with high Notch expression and activity[23].

In the present study, we sought to further classify the GBM neurosphere cultures previously used[23] by micro array analysis and assign them a possible Notch signature. In addition, the cultures were established as intracranial tumors in order to further determine the relevance of active Notch signaling for tumor formation. We found that neurosphere cultures with high endogenous Notch-1 expression, and high Notch pathway signature, formed tumors with a more infiltrative phenotype than neurosphere cultures with low Notch-1, and Notch pathway signature. However, in contrast to what was expected, we did not observe increased survival when we injected cells pretreated with

the Notch inhibitor DAPT as compared to a control treatment. We did, however, find that some of the tumors formed from DAPT treated cultures displayed lower levels of the stem cell marker Nestin and increased number of proliferative cells as well as abnormal vessels. We suggest that, despite indications of increased aggressiveness, bCSC targeted anti-Notch treatment in combination with traditional therapy might be feasible, as Notch inhibition possibly sensitizes the bCSC population to chemo- and radiation therapy by inducing them to differentiate.

MATERIALS AND METHODS

GBM neurosphere cultures

Establishment and characterization of the neurosphere cultures used in this study has previously been described[23, 24]. In short, neurosphere cultures were established from acutely dissociated patient derived subcutaneous xenograft tumors and maintained in NB-media (Neurobasal –A media supplemented with 1X B-27 Supplement, 1X L-glutamine, 10ng/ml Basic Fibroblastic Growth Factor (bFGF), 10ng/ml Epidermal Growth Factor (EGF), 1% Pen Strep (Penicillin-Streptomycin) (all from Invitrogen) and 10ng/ml Leukemia Inhibitory Factor (LIF, Chemicon)). The cells were cultivated in cell culture flasks (Nunc) in a humidified chamber with 5 % CO_2 at 37°C. Fresh media was added twice a week and spheres were mechanically dissociated at every passage. Neurosphere cultures used in the present study were: GBM_CPH029p7, NGBM_CPH036p7 and NGBM_CPH048p6. pX indicates the xenograft mouse passage from which the individual cultures were established. The prefix "N" refers to that the xenograft has been transplanted onto nude rats for a period to remove mouse hepatitis infection. For simplicity, the cultures will in the following be designated 029, 036 and 048 respectively.

Preparation of samples for micro array analysis

Neurosphere cells were dissociated and 1-1.5*10^6 cells were plated in 10ml NB-media in a petri dish (10cm Ø, Nunc) and treated the next day with 10µM DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-Butyl Ester (γ-secretase inhibitor (GSI) IX, Calbiochem) dissolved in dimethyl-sulfoxide (DMSO) hybri-max (Sigma)), equal volumes of DMSO for control or left untreated. 24 hours after treatment the cells were harvested and total RNA was extracted from cell pellets using QIAshredder columns and RNeasy Mini KIT (both from Qiagen) according to manufacturers' protocol. All RNA was DNase treated using the RNase-Free DNase Set from Qiagen.

Micro array analysis

RNA was amplified and labeled using the Ambion WT Expression Kit (Applied Biosystems) according to manufacturers' instructions. 250ng total RNA was used as input. The labeled samples were hybridized to the Human Gene 1.0 ST GeneChip array (Affymetrix). The arrays were washed and stained with phycoerytrin conjugated streptavidin (SAPE) using the Affymetrix Fluidics

Station® 450, and the arrays were scanned in the Affymetrix GeneArray® 3000 scanner to generate fluorescent images, as described in the Affymetrix GeneChip® protocol. Cell intensity files (CEL files) were generated in the GeneChip Command Console Software (AGCC) (Affymetrix). The data were modeled using the RMA (Robust Multichip Average) approach, followed by mean one step Probe Set summarization giving each gene a single expression value, all done using the Bioconductor affy library in the software package R.

Hierarchical cluster visualization of predefined Gene lists

A gene list representing genes involved in the KEGG_Notch_signaling_pathway was downloaded from the Molecular signatures Database v. 3.1 from the Broad Institute (MSigDB, http://www.broadinstitute.org/gsea/msigdb/index.jsp). Overlap between the downloaded gene list and the probes on the microarray was generated using the NetAffx database. Overlapping probe sets were selected based on overlap in gene symbol between annotated probes on the microarray and the gene list. Hierarchical cluster visualization was performed using dChip using Euclidean distance and average linkage clustering.

Intracranial growth of GBM neurosphere cells

Neurosphere cells were dissociated and $3*10^6$ cells were plated in 25ml NB-media in a culture flask (Nunc). DAPT was added the following day at a 10µM concentration and equal volumes of DMSO were added to the control. After seven days of treatment the cells were harvested by centrifugation (300xg), mechanically dissociated and re-suspended in warm NB-media at 10,000cells/µl. Parallel cell pellets were harvested and snap frozen in liquid N_2 for protein analysis (see below). 10µl cell suspension (100,000 cells) was injected into the brains of 6-9 weeks old female C.B-17 SCID mice: The mouse was anesthetized by *i.p.* administration of Hypnorm-Midazolam (1ml/100g body weight) and the head was fixed in a stereotactic frame (KOPF model 963, 926-B and 922: Better Hospital Equipment Corp). A short longitudinal incision was made in the scalp exposing the *calvarium*. Using a micro-drill, a burr-hole was made in the skull 1.5mm right of the *sutura saggitalis* and 0.5mm posterior to *bregma*. GBM neurosphere cells were injected at the depth of 2.0-2.5mm at a rate of 60nl/sec using a 100µl syringe with a 25-gauge needle (SGE100RN: World Precision Instruments, UK) placed in a micro infusion pump (Micro 4 pump and MicroSyringePump Controller: World Precision Instruments and KOPF model 1770-C: Better Hospital Equipment Corp). When injection was finished the needle was withdrawn after 1 min.

Bupivacain (0.2mg/100g body weight) and Lidocain (1mg/100g body weight) were administrated in the incision site for local anesthetic before the skin was closed with an Ethicon 5-0 prolene suture. 4-6 mice were injected in each group and tumor formation was monitored by frequently observing and weighing the animals. 1-2 additional mice were injected per group for *in situ* tumor visualization by CT/FET-PET scanning (FET: *O*-(2-[F]flouroethyl)-L-tyrosine) under Hypnorm-Midazolam anesthesia, but survival was used as the primary endpoint in this study. Mice were humanly euthanized when they showed tumor related symptoms such as a hunched position, bristly and greasy fur, lethargy, neurological signs and/or weight loss. Subsequently the brains were gently removed from the cranial cavity and fixed in 4% paraformaldehyde that after 24 hours was exchanged for EtOH 70%. For immunohistochemical (IHC) analysis the brains were sliced by coronally cutting the brain in the incision site and embedding the pieces in paraffin. From the block anterior to the incision site, 4µm histological sections were prepared for IHC (see below).

Protein purification and Western blotting

Whole cell lysates were prepared from cell pellets by sonication in ice-cold RIPA buffer (50 mM Tris-HCL (pH 7.4), 1 % NP40, 0.25 % Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with protease and phosphatase inhibitor mixture II and III (Calbiochem). Protein concentrations were determined using the BCA protein assay (Pierce, Herlev, Denmark) according to manufactures' instructions. For western blotting (WB) 50 µg protein was separated on a 4-12 % NuPAGE Bis-Tris gel and electroblotted onto nitrocellulose membranes (both from Invitrogen) according to manufacturers' protocol. The membranes were then blocked for 1 hour in 5% non-fat milk at room temperature (RT) and incubated with primary antibody diluted in 5% non-fat milk ON at 4ºC, followed by horseradish peroxidase (HRP) conjugated secondary antibodies for 1 hour at RT. Blots were developed using the SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and the UVP, BioSpectrum® AC Imaging System and VisionWorks®LS software (UVP). Primary antibodies: Goat polyclonal anti Notch-1 [S-20] (diluted 1:100, Santa Cruz #sc-23304), Rabbit polyclonal anti Hes-1 (diluted 1:2000, kindly provided by Dr. Tetsou Sudo, Toray Industries inc., Kamakura, Japan) and Rabbit monoclonal anti α-Tubulin [11H10] (diluted 1:1000, Cell Signaling #2125). Secondary antibodies: Rabbit polyclonal anti goat IgG (diluted 1:2000, Dako #P0217) and Swine polyclonal anti rabbit IgG (diluted 1:1000, Dako #P0449).

Immunohistochemistry

IHC was performed on formalin-fixed, paraffin-embedded tissue. For each of the three tumor-types in each of the two treatment groups, we stained histological sections (4μM) from three different mice with hematoxylin and eosin for normal histological evaluation and with antibodies detecting four different molecular markers. All IHC stainings were performed manually. Briefly, formalin-fixed and paraffin-embedded slides were melted for one hour at 60ºC, followed by deparaffination in xylene and rehydration. Endogenous peroxidase activity was quenched by treating with 0.3% hydrogen-peroxide in water for 30 minutes. Antigen retrieval was performed by immersing the sections in water bath containing citrate buffer (DAKO, Denmark) for 30 minutes at 95°C. Subsequently, the slides were blocked for 20 min in PBS with 2% horse serum before incubation with primary antibody diluted in blocking buffer over night at 4°C followed by biotinylated universal secondary antibody (anti-rabbit and -mouse IgG) and horseradish peroxidase (HRP) conjugated Avidin/Biotin Complex (ABC) reagent (secondary antibody and ABC reagent were both diluted 1:50 and incubated for 30-60 minutes at RT, Vector Kit #PK-6200). The signal was developed by Diaminobenzidine (DAB, BioGenex #HK153-5KE) for 5 minutes. Finally the sections were counterstained with Mayer´s haematoxylin, dehydrated with increasing concentrations of ethanol and mounted with DPX (Sigma-Aldrich). For negative controls the primary antibodies were omitted. Primary antibodies used: Nestin (diluted 1:10,000, Millipore #NG1853940), Notch-1 (diluted 1:200, Cell Signaling #3608), CD31 (detecting both human and murine CD31, diluted 1:50, Abcam # ab28364) and Ki-67 (diluted 1:100, Abcam # ab8191). Light microscopy of the IHC sections was carried out using the Olympus BX51 microscope, the Olympus D71 camera and the Cell^A 2.5 (Build 1163) Olympus Soft Imaging Solutions GmbH software.

RESULTS

Micro array analysis and Notch signature

We have previously categorized the 029 and 036 neurosphere cultures as having high endogenous Notch-1 expression and Notch pathway activation, as indicated by high Hes-1 expression, when compared to the 048 culture which we have classified as having low Notch-1 expression and activation. We have also shown that Notch inhibition by DAPT treatment did not affect the 048 culture to the same degree as the 029 and 036 cultures[23]. To elaborate this, we have performed a global genome expression analysis and used this to establish a Notch signature of the three cultures. The expression of selected Notch pathway components is shown in Figure 1A. (For the expression of all Notch pathway components included in the "KEGG_Notch_signaling_pathway" gene set, see Supplementary Figure: S1). As displayed in Figure 1A, with regard to the majority of the Notch components shown, the 048 culture differs from the 029 and 036 cultures. Notably, the Notch-1, -2 and -3 receptors, the ligands Delta-like (Dll) -1, Jagged-1 and -2, and the downstream targets Hes-1 and Hey-1 are all down regulated in 048 compared to 029 and 036, indicating a higher activity of the Notch pathway in the two latter cultures. Moreover, the ligands Dll-3 and -4 were up-regulated in the 036 culture as compared to the 029 and 048 cultures.

We then analyzed the expression of the same genes in cultures treated with 10μM DAPT or equal volumes of DMSO for 24 hours. As seen in Figure 1B Hes-1 and Hey-1 were down regulated upon DAPT treatment in all three cultures verifying successful Notch inhibition. Furthermore the γ-secretase sub-unit PSENEN was likewise down regulated in all cultures, while the DLL-1 was up regulated and DLL-3 and Jagged-2 showed the same tendency.

Different neurosphere cultures give rise to intracranial tumors with different features, but all display GBM characteristics

To extend our *in vitro* findings, that the different patient derived neurosphere cultures display different characteristics such as growth- and expression patterns (as presented above and in[23] we engrafted the neurosphere cells *in vivo* in order to examine whether the cultures also formed dissimilar tumors. When injected into the brains of immunocompromised mice, all neurosphere cultures formed intracranial tumors verifying their tumorigenic potential. However, there was a considerable difference in the time from injection to that the mouse had to be euthanized, between the cultures. In Figure 2A, the survival in weeks of mice injected with control treated 029, 036 or 048 cells is presented. Mice injected with 036 cells survived the longest namely 29.60 weeks (95%

CI: 20.88-38.32), while mice injected with 029 neurosphere cells survived approximately 17.20 weeks (95% CI: 15.58-18.82) and mice injected with 048 cells had the shortest survival at 6.25 weeks (95% CI: 4.73-7.77). Furthermore, mice injected with 036 cells showed the highest variability in survival, from 19 to 38 weeks, while mice injected with 048 cells survived from 5-7 weeks. The histological appearance of the tumors also varied greatly, especially when comparing the 029 and 036 tumors with the 048 tumors. Representative H&E sections of tumors formed from control treated neurosphere cultures are displayed in Figure 2B. In general, the 048 tumors were mostly uniform in appearance with a well defined border between the tumor and the surrounding normal brain parenchyma whereas the 029 and 036 tumors were much more disorganized with no clear border and especially the 036 tumors moreover tended to home to the ventricles (For additional H&E pictures, see Supplementary Figure S2). Using CT/FET-PET scanning we were furthermore able to detect *in situ* tumor formation in mice injected with cells from each of the three cultures and it was as such possible to follow tumor growth in real time. In Figure 2C, coronal views of the CT/FET-PET scannings of one representative mouse at week 1, 4, 5 and 6 after injection with 048 neurosphere cells are presented. The mouse was euthanized at week 7. Histological GBM hallmarks were evident in tumors from all of the neurosphere cultures used (029, 036 and 048) and representative H&E stainings showing mitosis, invasion, necrotic areas and excessive vascularization are shown in Figure 2D. It should be noticed that the vessels did not exhibit an abnormal/malignant phenotype. Finally, by evaluating the expression of the Notch-1 receptor in the intracranial tumors using IHC staining, we found that the 048 tumors tended to display a weaker coloration when compared to the 029 and 036 tumors indicating a lower expression of Notch-1 in these tumors, which is in good correlation with our *in vitro* observations. Representative pictures are displayed in Figure 2E.

Notch inhibition in neurosphere cells does not hamper intracranial tumor formation and growth

We have previously demonstrated that pre-treating the 029 and 036 cells with DAPT almost abolished their ability to form clonogenic colonies in soft agar possibly as a consequence of slowing of the cell cycle, whereas no effect was seen on the 048 cells [23]. To determine if inhibition of Notch signaling prior to xenograftment also affected the ability of intracranial tumor growth, we treated the 029, 036 and 048 neurosphere cells with 10μM DAPT *ex vivo* for seven days and then injected 100,000 viable cells orthotopically into the brains of C.B-17 SCID mice. DMSO was used as a control treatment. Each individual mouse was monitored frequently during the experiment and was

euthanized when it showed tumor related symptoms or considerable weight loss (Supplementary Figure S3).

To test if Notch signaling was inhibited in the injected cells at the time of intracranial injection, the Notch-1 protein level and the activity of the Notch pathway, as assessed by Hes-1 expression were analyzed in DAPT and control treated samples that were generated in parallel to the cells injected. As expected, Notch-1 and Hes-1 were expressed at higher levels in the control treated 029 and 036 cultures as compared to the 048 culture (Figure 3A), which supports our previous findings that the 029 and 036 cultures have a high Notch activation level as compared to the 048 culture[23]. Furthermore, a prominent down regulation of Hes-1, indicative for Notch pathway inhibition, could be detected in 029 and 036 DAPT treated samples when compared to the control, whereas the down regulation was not as evident in the 048 culture. We thus concluded that Notch signaling was inhibited in the DAPT treated cells that were intracranially injected. However, as displayed in Figure 3B and D, there was no statistical difference in survival between mice injected with DAPT treated 029 cells when compared to DMSO ($p= 0.32$) or DAPT treated 048 cells when compared to DMSO ($p=0.22$), indicating that there was no major difference in intracranial tumor growth between the control and DAPT treated groups. There was, however, a tendency towards decreased survival for mice injected with DAPT treated 036 cells when compared to DMSO treated 036 cells ($p=0.047$) (Figure 3C).

Histological appearance differed between tumors

Three tumors from each group were evaluated by histological analyses. As could be visualized by H&E staining, there was a tendency towards that DAPT treated 036 cells formed highly vascularized tumors with large voluminous vessels (representative H&E pictures are shown in Figure 4). Three out of three 036 DAPT tumors evaluated demonstrated this phenotype which was also observed in one out of three 029 DAPT tumors. No obvious difference was observed between 048 DMSO and DAPT tumors (additional pictures are shown in supplementary Figure S2). The stem cell marker Nestin, was evaluated by staining intensity, distinguishing between if all tumors cells were highly positive or if they in general displayed a more pale coloration, or if there existed areas within the tumor tissue that were negative (marked "+" and "(+)" respectively in Table 1). This way we found that all DMSO tumors, regardless of origin showed high expression of Nestin in all tumor cells. The same was the case for all 048 DAPT tumors. In contrast three out of three 029 DAPT tumors and one out of three 036 DAPT tumors showed a decreased expression of Nestin,

either by displaying a general weaker staining in the tumor cells (exemplified by 029 in Figure 4H) or by containing areas with almost no Nestin positive cells (exemplified by 036 in Figure 4J).

The proliferation marker Ki-67 was analyzed by evaluating the fraction of Ki-67 positive nuclei. In Table 1 less than 25% positive nuclei is annotated "+" and more that 25% is annotated "++". As such, we found no difference in the fraction of proliferative cells between 029 DMSO and DAPT tumors as well as in 048 DMSO and DAPT tumors. In contrast the 036 DMSO tumors displayed the lowest fraction of proliferative cells and surprisingly, this fraction increased in the 036 DAPT tumors.

Finally, the endothelial cell marker CD31 was assessed by observing the presence of abnormal vessels. No abnormal vessels in the section was assigned "-", few abnormal vessels (1-10) was assigned "(+)" and high density of abnormal vessels was assigned "+". None of the DMSO tumors displayed any abnormal vessels (Table 1). Abnormal vessels were observed in one out of three of the 029 DAPT tumors and three out of three of the 036 DAPT tumors. Furthermore, two out of three 048 DAPT tumors showed a few small abnormal vessels.

DISCUSSION

In the latter years researchers have attempted to sub-type GBM based on global gene expression and proteomics[19, 25, 26]. In the future this sub-typing is thought to assist clinicians in stratifying GBM patients to the most optimal treatment as has proven possible with MGMT methylation and temozolomide (TMZ, Temodal®) treatment[27]. As such, it is believed that the different sub-types display molecular hallmarks that are of specific importance for each sub-type. Accordingly, the Notch signaling pathway has been associated with the classical sub-type[19]. We have previously shown that the 029 and 036 patient derived xenograft tumors and thereof derived neurosphere cultures could be characterized as having high Notch-1 and Hes-1 expression while 048 tumors and cultures were characterized as having low Notch-1 and Hes-1 expression [23], indicating that they belong to different GBM sub-types. In the present study we have sought to elaborate this finding, by analyzing the global gene expression pattern of the three cultures and further study the effect of Notch inhibition on intracranial tumor growth. Here, we find that the 029 and 036 cultures show greater correlation to each other with regard to expression of a panel of Notch pathway components than compared to the 048 culture. Components that were less expressed in 048 as compared to 029 and 036 were among others the Notch-1-3 receptors and the Notch target genes Hes-1 and Hey-1, indicating a lower Notch signaling pathway activity. On the other hand, the Notch-4 receptor and the transcriptional target Hes-5 were up regulated in 048 as compared to 029 and 036. The Notch-4 receptor has been implicated in the formation of mouse mammary tumors[28] and in maintenance of stem cells in breast cancer[29] and it has furthermore been suggested as a therapeutic target in triple negative breast cancers[30]. However, the function of Notch-4 in GBM remains undetermined. As Hes-5 is expressed in the 048 culture this indicates that the Notch signaling pathway is active also in this culture although the effects of inhibiting it are less pronounced as compared to 029 and 036[23]. Moreover, Hes-5 seems to be up-regulated in 048 upon DAPT treatment, while down-regulated in 029, suggesting that Hes-5 expression is regulated by different means in the 048 culture. However, it is worth mentioning that Hes-5 knock-outs during embryogenesis are less severe than Hes-1 knock-outs, indicating an inferior role for Hes-5 in neural stem cells (reviewed in Fischer *et al*. (2007)[31]). One might argue, that expression of the γ-secretase sub-unit PSENEN and the ADAM metalloprotease ADAM-17 in 048 also indicates high Notch pathway activation. However, these are not exclusively modulators of Notch pathway activity. E.g. γ-secretase is also involved in the cleavage of among others the beta-amyloid precursor protein (APP)[32]. Taken together, the three cultures examined in the present project display different activation of the Notch

signaling pathway, with the 029 and 036 most likely representing cultures with elevated Notch signaling signature as compared to the 048 culture.

When the cells were injected into the brains of SCID mice and allowed to form tumors, the histological appearance of the tumors also varied with the 029 and 036 tumors displaying a more diffuse and infiltrative growth pattern as compared to the 048 tumors that formed large, well bordered tumors. These observations could indicate that an endogenous high Notch activity leads to a more invasive and thus aggressive phenotype. In line with this, it has been suggested that the level of Notch-1 expression increases with tumor grade in gliomas[33] and that Notch promotes migration and invasiveness of glioma cells, possibly through activation of β-catenin and NFκB signaling[34]. However, considering the fact that the 048 neurosphere cells formed tumors much faster than especially the 036 cells, one might speculate that massive well defined tumors are the result of fast growth and expansion of the tumor cells that displace the normal brain parenchyma simply by mass effect, while infiltrative growth is a slower process due to degradation of tissue barriers. This is supported by observations that most 048 tumors were clearly detectable macroscopically both by the formation of a doomed head and by a heavily enlarged right hemisphere indicating a large mass within the brain that had dislocated the scalp. In contrast, mice injected with 029 and especially 036 cells, rarely displayed these objective signs of a tumor mass.

We have previously shown that the 029 and 036 neurosphere cultures were sensitive to Notch inhibition as exemplified by reduced *in vitro* clonogenic growth potential upon pretreatment with the Notch inhibitor DAPT[23] when compared to the DMSO control. However, when the pretreated cells were injected into the brains of SCID mice, no increased survival was observed when compared to tumor formation from control treated cells. Thus pretreatment of the neurosphere cells with DAPT did not reduce tumor formation, indicating that it is not possible to eliminate all tumor forming cells by Notch inhibition. Surprisingly, DAPT treatment of the 036 cells actually seemed to decrease survival and thus form tumors more rapidly than the control treated 036 cells. This discrepancy might reflect that, when we previously performed the *in vitro* clonogenic assay, we continued the treatment throughout the assay period, whereas when the cells were injected into the mice, DAPT was removed and the treatment ceased. Although Notch inhibition might not have been halted immediately after DAPT removal, it is most likely that the Notch signaling was restored *in vivo*, which has also been observed by others[18]. The lack of positive effect from Notch treatment on survival is in contrast to previous reports showing that GSI treatment prior to engraftment significantly prolonged survival of the mice[18]. Fan and colleagues suggested that this was due to

ablation of bCSC by Notch inhibition. They did, however, not observe any difference between treatment and control groups by histological evaluation of H&E sections[18]. In the present study, we found that DAPT treatment of the 029 and especially the 036 neurosphere cells resulted in tumors with large voluminous vessels. When evaluating the endothelial cell marker CD31 we found that these vessels tended to be disorganized and abnormal. Taken together, these results might lead to speculations that *ex vivo* Notch inhibition in neurosphere cells with high endogenous Notch activity selects for a more angiogenic cell phenotype *in vivo*. In line with this, it has been shown that inhibition of the Notch ligand Dll-4 leads to increased angiogenesis, although the vessels are non-functional[35, 36] and it is moreover been speculated if targeting Dll-4 could improve anti-angiogenic therapy by triggering excessive but malfunctional angiogenesis[36, 37].

Regarding normal neural stem cells, it has been shown that the Notch pathway is essential for maintaining the undifferentiated phenotype and that inhibition of the pathway leads to differentiation[38-40]. If active Notch signaling in the same way plays a role for maintenance of the immature state of the bCSC population in the culture but is not essential for the growth of GBM neurosphere cells *in vivo*, one might speculate that Notch inhibition merely leads to differentiation of the bCSC cells as it previously has been demonstrated[16, 17, 41], rather than killing them as others have suggested[18]. In line with this, it has been demonstrated that some GSIs, including DAPT are unable to kill bCSC[42]. As such, it could be speculated that the DAPT treatment in our study targeted the bCSC population by differentiating them and as such, the cells injected into mice were more differentiated progenitor cells that were still able to form tumors. This is supported by the findings that some of the 029 and 036 tumors established from DAPT treated cells seemed to have lower levels of the stem cell marker Nestin, evaluated by IHC. This decrease of Nestin positive cells upon Notch inhibition has also been reported *in vitro*[41] and Nestin has moreover been proposed as a direct transcriptional target of the Notch pathway activity in GBM[43]. Furthermore, in the case of 036, the DMSO tumors displayed fewer proliferating cells than the DAPT tumors which correlates with suggestions that progenitor cells proliferate faster than the more immature stem cells[44-46]. In addition, it has been demonstrated that bCSC can transdifferentiate into endothelial cells[47], and an intriguing thought might be that Notch inhibition induces differentiation of at least some of the bCSC down an alternative lineage, namely towards endothelial cells, which might explain the appearance of malignant vessels in the tumors generated from DAPT treated neurosphere cells. Taken together, these data suggest that Notch inhibition by DAPT treatment leads to a more differentiated phenotype with a higher proliferative index, abnormal vasculature and perhaps even

decreased survival. Apart from differentiation, these are all indications of the Notch pathway being a tumor suppressor. Indeed it has been demonstrated that Notch is considered a tumor suppressor in different cancer types, as exemplified in the embryonal brain tumor medulloblastoma. Here Notch-1 and Notch-2 were showed to have opposite effects with Notch-1 acting as a tumor suppressor and Notch-2 as an oncogene[48]. However, GSI treatment targets all four Notch receptors. Moreover, if inhibition of Notch signaling partly induces differentiation of the bCSC population into faster proliferating progenitor cells and cells with endothelial cell function, and if considering the implications that bCSC can be accounted for the chemo- and radio resistance seen in GBM patients, maybe a combination of traditional therapy targeting the highly proliferative cancer cells together with Notch inhibition that differentiate and thus sensitize the bCSC population to the traditional treatment[49] would be feasible. However, this is highly speculative and future studies will need to verify this hypothesis. Nevertheless, based on the initial results of this manuscript, we believe that the Notch signaling pathway presents a potential target for future anti-bCSC treatment and that a regimen that includes Notch inhibition would improve the therapy for GBM patients.

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FIGURE LEGENDS

Figure 1. Gene expression analysis revealed different Notch signatures between the GBM neurosphere cultures investigated. A) Expression of selected Notch pathway components in the 029, 036 and 048 neurosphere culture. B) Expression of the same components in the same neurosphere cultures treated with 10μM DAPT or equal volumes of DMSO for 24 hours. In both A) and B) the expression level is relative to the mean expression of the respective gene in the three or six samples respectively.

Figure 2. GBM neurosphere cells forms tumors with different phenotypes when injected into mouse brains. A) Survival in weeks of mice injected with 029, 036 or 048 neurosphere cells treated with the control substance DMSO for 1 week prior to injection. Column statistics were generated using the GraphPad Prism 4 software (GraphPad Software, http://www.graphpad.com). B) Representative H&E staining of the tumors displayed in A). C) CT/FET-PET scans of a mouse injected with DMSO treated 048 cells at week one, four, five and six after injection. D) Representative H&E pictures of the intracranial tumors showing characteristics of high grade gliomas i.e. mitosis and invasion and GBM specific hallmarks i.e. necrosis and excessive vasculature. Green arrows indicate mitotic cells. E) Representative immunohistochemical stainings of the Notch-1 receptor in tumors formed from 029, 036 and 048 neurosphere cells, respectively. Scale bar shows 50μm.

Figure 3. No survival benefit for mice injected with DAPT treated GBM neurosphere cells. A) Protein expression of the Notch-1 receptor and the Notch transcriptional target Hes-1 in GBM neurosphere cells treated with DMSO for control (-) or 10μM DAPT (+) for seven days, prior to intracranial injection. B-D) Kaplan-Meyer plots showing the fractionated survival of mice injected with B) 029, C) 036 or D) 048 cells pretreated for one week with either 10μM DAPT or equal volumes of DMSO. p-values represents the comparison of survival curves by log-rank test in the GraphPad Prism 4 software.

Figure 4. Histology and expression of the stem cell marker Nestin, the proliferative marker Ki-67 and the endothelial cell marker CD31 differed between tumors formed from DAPT and DMSO treated neurosphere cells. A-F) H&E of representative tumors from each treatment group of the three tumor-types. Scale bar shows 1mm. G-L) Immunodetection of the stem cell marker

Nestin. Positive cells stain dark brown in the cytoplasm. Scale bar shows 200µM. M-R) Immunodetection of the proliferative marker Ki-67. Positive nuclei are stained dark brown. Scale bar shows 100µM. S-Y) Immunodection of the endothelial marker CD31. Normal vessels are shown as strait brown stripes in the section, while abnormal vessels are detected as disorganized vessels often with multiple endothelial cells constituting the vessel wall. Scale bar shows 100µM. The pictures are not by certainty from the same tumor.

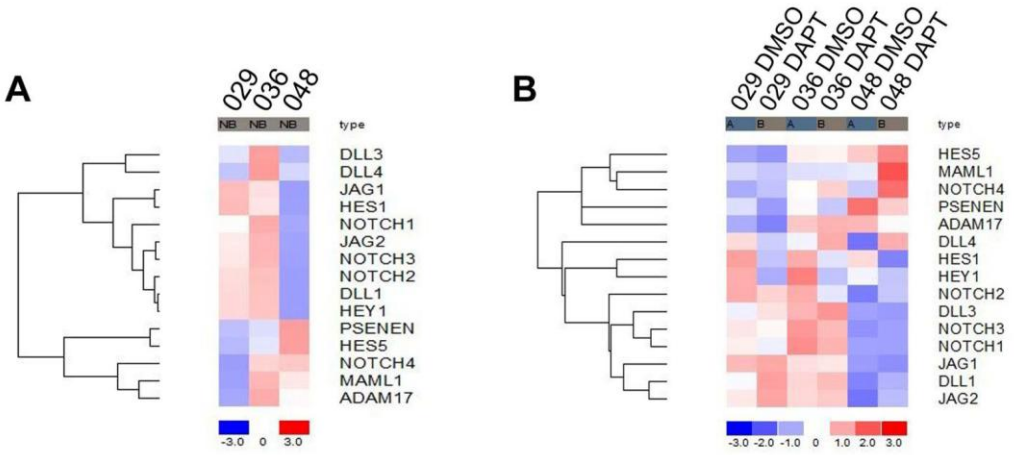
Table 1: Evaluation of Nestin, Notch-1, Hes-1, CD31 and Ki-67 expression in brain sections from mice injected intracranial with DAPT or DMSO treated neurosphere cells. For each of the three tumor-types in each treatment group, we stained histological sections of three different mice. Markings represent for Nestin: "+" = all tumors cells were highly positive for Nestin, "(+)" = either a more pail coloration of the tumor tissue in general or areas within the tumor tissue that are negative for Nestin. For Ki-67: "+" = less than 25% positive nuclei and "++" = more than 25% positive nuclei. For CD31: "-" = no abnormal vessels, "(+)" = between 1 and 10 abnormal vessel in the section and "+" more than 10 abnormal vessels in the section.

Supplementary Figure S1: Gene expression analysis revealed different Notch signatures between the GBM neurosphere cultures investigated. Expression of all Notch components from the "KEGG Notch signaling pathway" gene set downloaded from the "Molecular Signature Database v3.1" on the *http://www.broadinstitute.org/gsea/msigdb/index.jsp* webpage. The expression level is relative to the mean expression of the respective gene in the three or six samples respectively.

Supplementary Figure S2: H&E pictures of the tumors evaluated by IHC. Scale bar shows 1mm.

Supplementary Figure S3: Weight curves of the individual mouse throughout the experiment period. Each individual mouse was monitored frequently during the experiment and was euthanized when it showed tumor related symptoms or considerable weight loss.

Figure 1


A
029
036
048
NB NB NB
type
DLL3
DLL4
JAG1
HES1
NOTCH1
JAG2
NOTCH3
NOTCH2
DLL1
HEY1
PSENEN
HES5
NOTCH4
MAML1
ADAM17
-3.0 0 3.0
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029 DMSO
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type
HES5
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PSENEN
ADAM17
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HEY1
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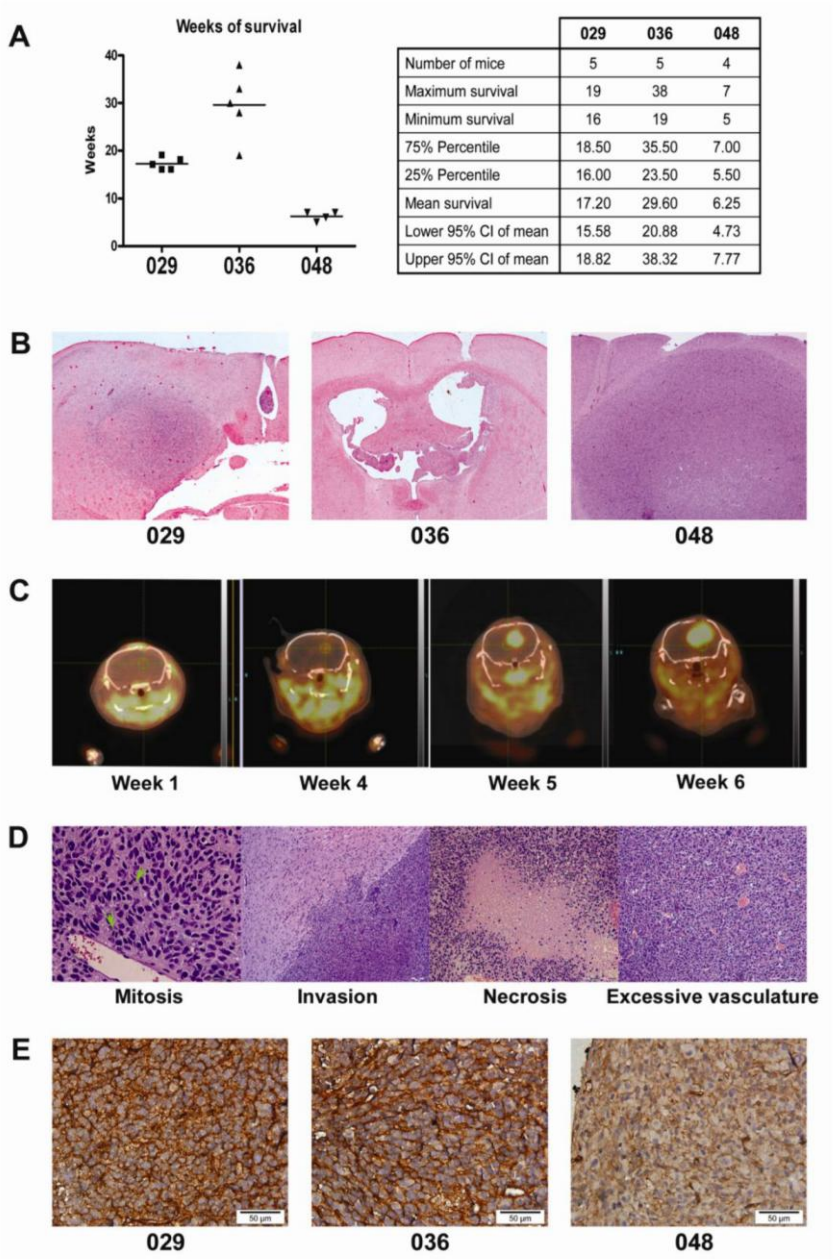
Figure 2



	029	036	048
Number of mice	5	5	4
Maximum survival	19	38	7
Minimum survival	16	19	5
75% Percentile	18.50	35.50	7.00
25% Percentile	16.00	23.50	5.50
Mean survival	17.20	29.60	6.25
Lower 95% CI of mean	15.58	20.88	4.73
Upper 95% CI of mean	18.82	38.32	7.77

Figure 3

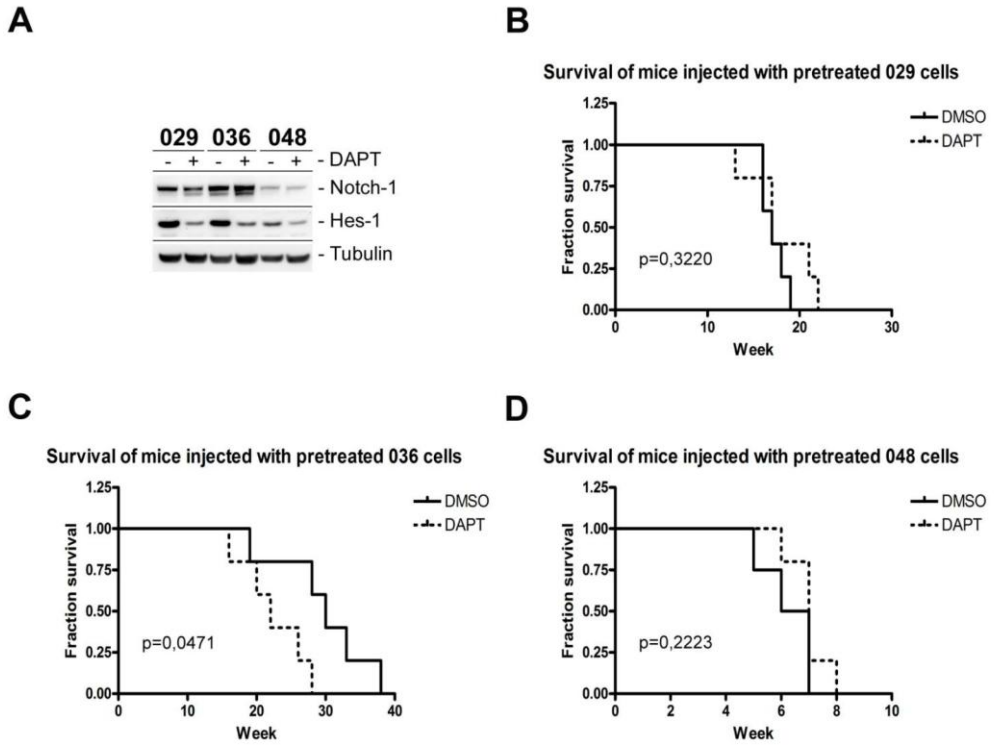

A
029 036 048
- + - + - +
- DAPT
- Notch-1
- Hes-1
- Tubulin
B
Survival of mice injected with pretreated 029 cells
DMSO
DAPT
Fraction survival
p=0,3220
Week
C
Survival of mice injected with pretreated 036 cells
DMSO
DAPT
Fraction survival
p=0,0471
Week
D
Survival of mice injected with pretreated 048 cells
DMSO
DAPT
Fraction survival
p=0,2223
Week

Figure 4

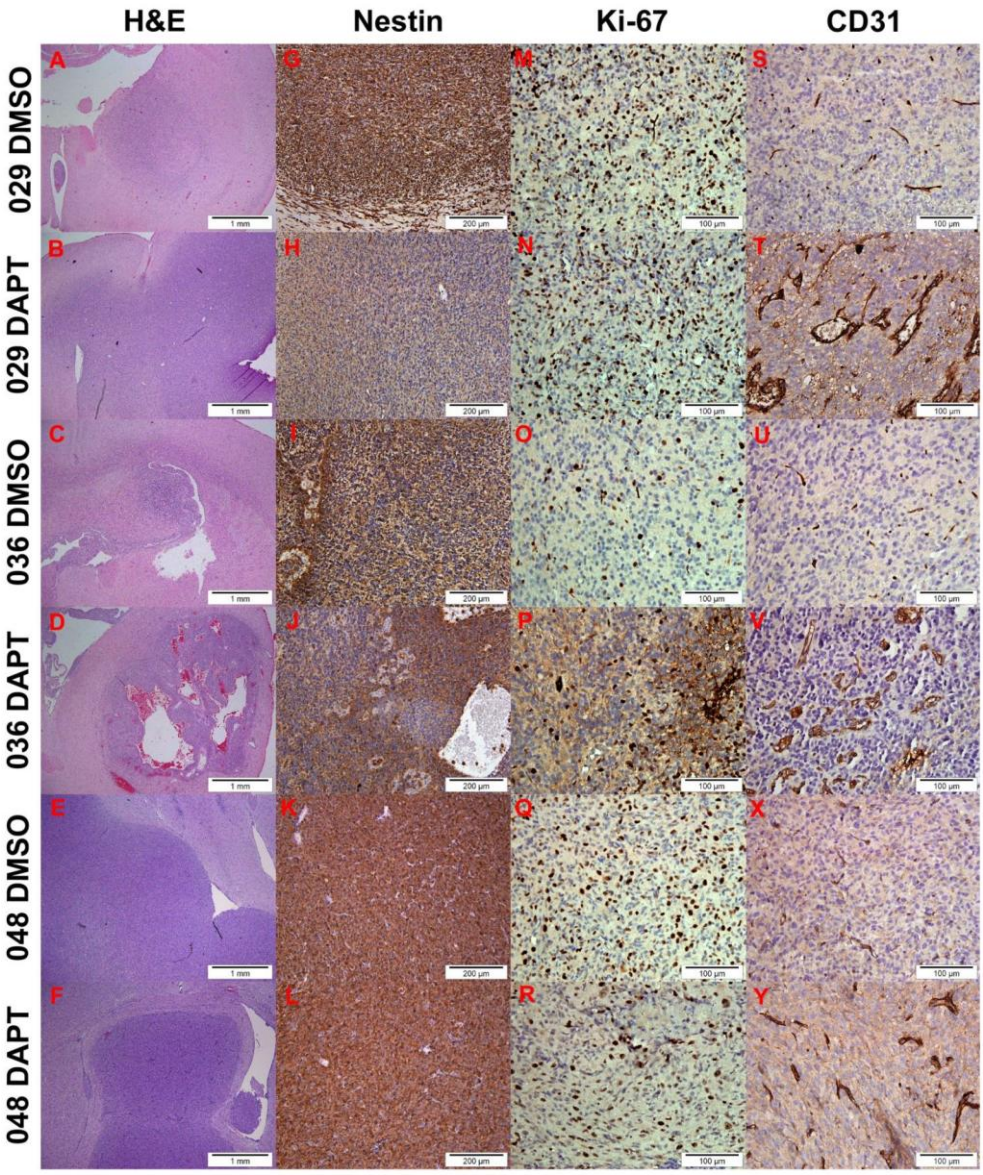

H&E
Nestin
Ki-67
CD31
029 DMSO
029 DAPT
036 DMSO
036 DAPT
048 DMSO
048 DAPT
A
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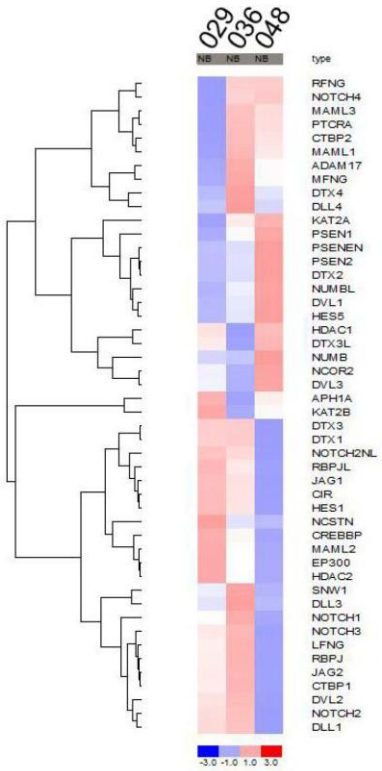
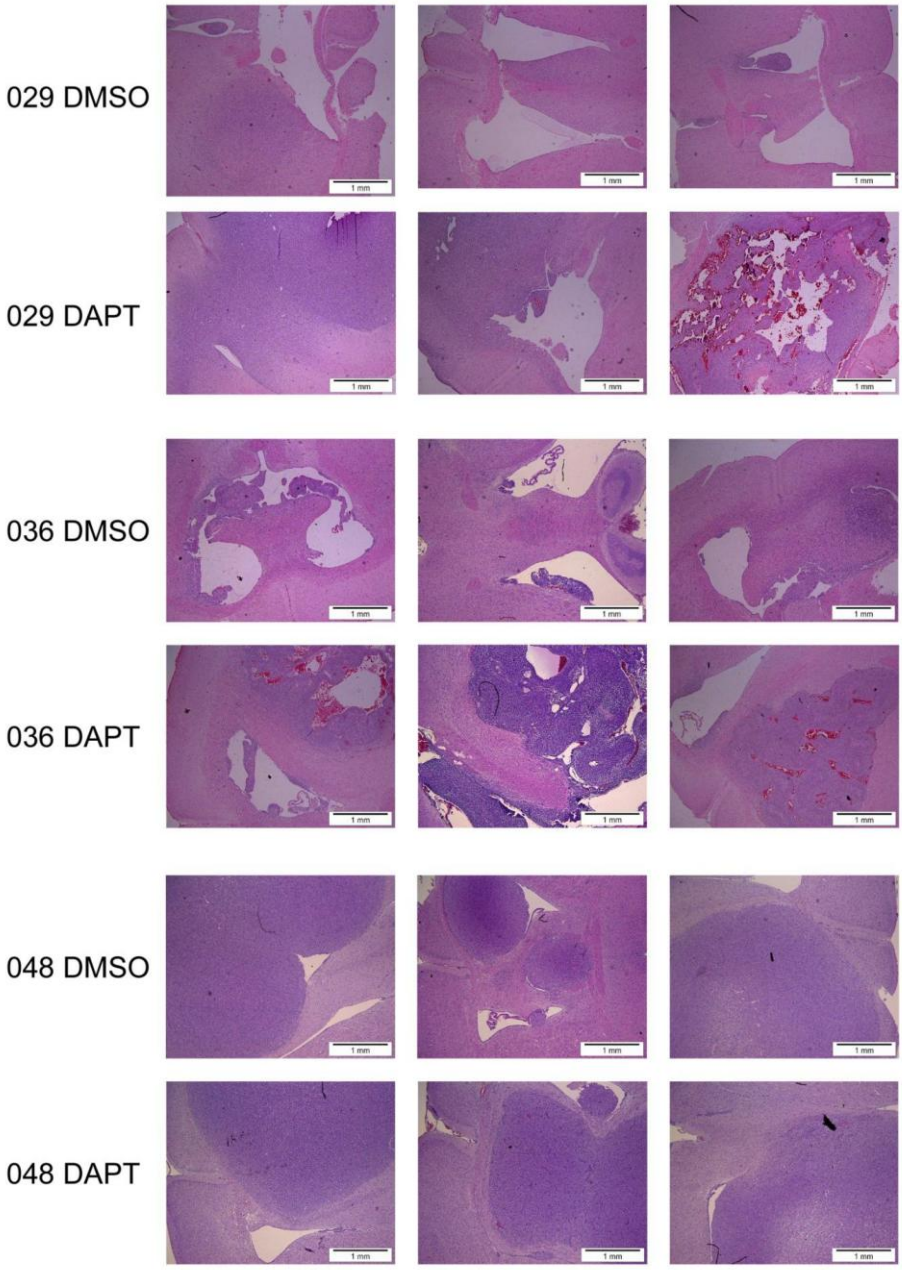
Table 1

Table 1: Expression of different markers in orthotopic GBM tumors derived from GBM neurosphere cells pretreated with 10µM DAPT or DMSO for control				
Cells injected	**Pretreated with**	**Nestin**	**Ki-67**	**CD31**
029	DMSO	+	++	-
		+	N.A.	-
		+	++	-
	DAPT	(+)	++	-
		(+)	++	-
		(+)	++	+
036	DMSO	+	+	-
		+	+	-
		+	+	-
	DAPT	(+)	++	+
		+	++	+
		+	++	+
048	DMSO	+	++	-
		+	N.A.	N.A.
		+	++	-
	DAPT	+	++	-
		+	++	(+)
		+	++	(+)

Supplementary Figure S1



Supplementary Figure S2



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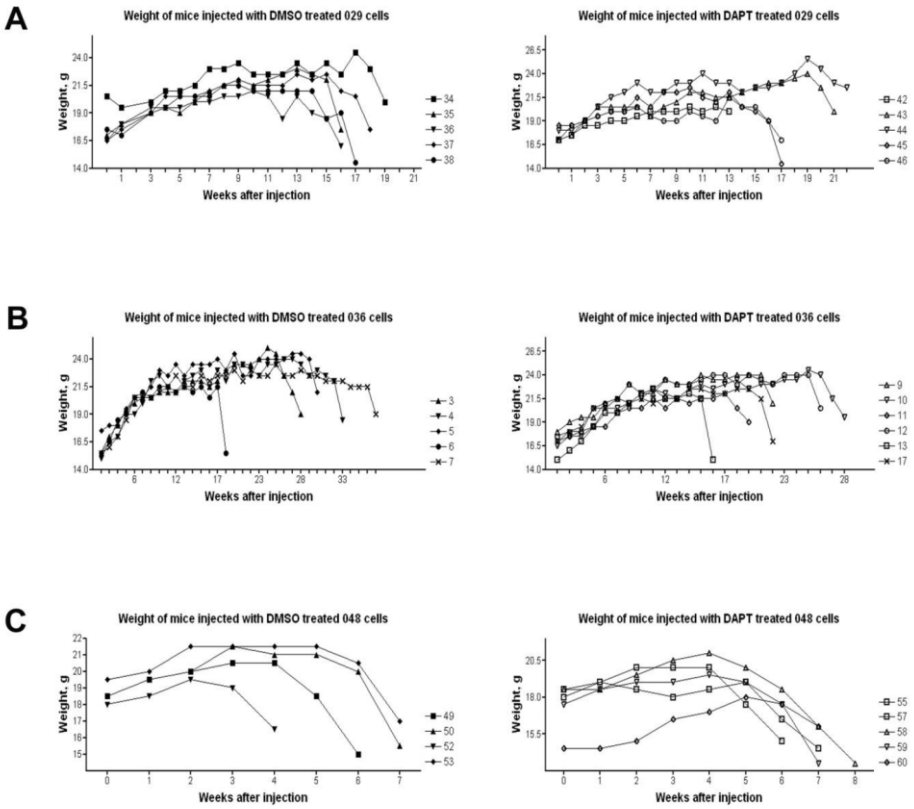
Supplementary Figure S3



8. Summarizing discussion and perspectives

It has repeatedly been demonstrated that tumor cells with stem cell-like properties can be cultured from human GBM by using conditions that selects for the expansion of NSC[3,11,71]. It is moreover well established that EGFR and Notch signaling plays an important role in the balance between the normal NSC population and its differentiated progeny and increasing evidence suggests that these pathways also are involved in the maintenance of the bCSC population in GBM. This PhD project adds to the existing knowledge of the functional role of EGFR and Notch signaling in GBM derived bCSC stem cell-like features and tumorigenic properties.

An overview of the naming of patient tumors and corresponding xenografts and cultures established in manuscript I and investigated in manuscripts II, III and IV are presented in Table 1.

8.1 Establishing and characterizing an *in vivo*/*in vitro* model of glioblastoma multiforme

A good experimental model, for any human disease, is a model that resembles the human condition as close as possible. *In vitro* models are popular as they are easy and fast to work with, fairly cheap and very favorable when screening for potential new targets and when studying specific molecular pathways and (their role in) cellular phenotypes hypothesized to be important for e.g. a specific tumor type. *In vivo* models are more laborious, but they offer valuable insight into tumor-host interactions and a platform for testing the potential of promising new therapeutic targets. The lack

Table 1. Naming of the patient tumors and corresponding xenografts and neurosphere cultures established in **MANUSCRIPT I** and investigated in **MANUSCRIPTS II**, **III** and **IV**, unless otherwise stated.

Patient tumor	Subcutaneous xenografts	Neurosphere culture	Manuscript
GBM_CPH017	NGBM_CPH017p4	017p4	III
GBM_CPH029	GBM_CPH029p7	029p7 or 029	III, IV
	NGBM_CPH029p5 2H	029p5	III
GBM_CPH036	NGBM_CPH036p6	036p6	N.A.
	NGBM_CPH036p7	036p7 or 036	III, IV
	NGBM_CPH036p8	036p8	III
	NGBM_CPH036p15	036p15	III
GBM_CPH047	NGBM_CPG047p2 1V	047p2	III
	NGBM_CPG047p2 1H	047p2 1H	N.A.
	NGBM_CPH047p3m1	047p3 or 047	II
	NGBM_CPH047p3m2	047p3m2	N.A.
	NGBM_CPH047p4m1	047p4	N.A.
GBM_CPH048	NGBM_CPH048p4	048p4	N.A.
	NGBM_CPH048p5	048p5	N.A.
	NGBM_CPH048p6	048p6 or 048	III, IV
	NGBM_CPH048p7	048p7	III

The p-number immediately following the patient tumor number refers to the mouse passage from which the neurosphere culture has been established from. The prefix "N" refers to the tumor has been passaged over nude rats to remove mouse hepatitis. N.A. = not further investigated

of a good and reliable experimental model for GBM has been an issue especially when exploring tumor specific targeted therapies as traditional serum-containing *in vitro* culturing methods of GBM cells have been shown to result in loss of important tumor hallmarks. E.g. EGFR amplification and mutations are lost already after few passages in serum-containing media and these cultures do as such not exhibit very good resemblance to clinical GBM tissue[3,55]. As an alternative, scientists have used commercial glioma and GBM cell lines that have been modulated to express these hallmarks (as discussed in section 2.1.1). However, these cell lines are likewise grown in serum-containing media and do not resemble patient tumor tissue[3].

In recent years it has become clear that GBM can be sub-typed further than into primary and secondary GBM, and that this sub-typing most likely has an influence on prognosis and treatment outcome, as exemplified by MGMT methylation status and gene expression profile, discussed in section 2.1. Thus, the few commercially available cell lines are not able to embrace the broad diversity found within the human disease. As a consequence, great effort has been put into developing new and more reliable experimental *in vitro* GBM models that represent as many aspects of human GBM as possible. During the past decade, the serum-free neurosphere culture method, outlined in section 2.2.1, has gained increasing acceptance. It has been developed based on the findings that a population of cancer stem-like cells seems to drive the malignant features of leukemia[100] and from experience in working with normal NSC (reviewed in Chaichana *et al.* (2006)[68]). This led to the identification of a stem cell-like brain cancer cell for the first time in 2002 by Ignatova *et al.*[11] and in the following years several reports of a tumorigenic brain cancer stem-like cell population has been published[70-72,79]. It has subsequently been demonstrated that the serum-free GBM cultures exhibit characteristics of normal NSC, show better resemblance to the patient tumor than serum-cultured GBM cells and furthermore have tumorigenic potential when transplanted onto immunocompromised mice[3,56,79]

8.1.1 Establishing an experimental GBM model

We have exploited the serum-free approach to establish an experimental *in vivo/in vitro* GBM model that maintains hallmarks of the original patient tumor (**MANUSCRIPT I**). The reason for using a subcutaneous xenograft intermediate station was to assure continuous availability of fresh (and frozen) tumor tissue for establishing both new xenograft tumors as well as *in vitro* cultures for our experimental GBM model representing different patient tumors. This way we were also able to test if the tumor tissue available continued to express the patient tumor hallmarks of interest throughout xenograft passaging. A GBM patient tumor is commonly referred to as a very heterogeneous tumor, with areas of necrosis and vascular proliferation being GBM hallmarks. If the tumor biopsy available for scientific purposes was mostly necrotic, it would make it difficult to establish it in any experimental model, which might have been the case in **MANUSCRIPT I** were only 50% of patient tumors were successfully established as xenograft tumors. It has furthermore been suggested that the gene expression profile within one tumor can vary from one area to another[53] and as such, the biopsy specimen we had was not by certainty a good representative for the tumor as a whole. However, if bCSC are present in the biopsy, they should be able to regenerate a representative patient tumor xenograft as the cancer stem cell hypothesis states and which has been reported in previous studies[3,56]. Our results support these findings as we have shown that the expression of

EGFR and EGFRvIII, as detected in the original patient tumor, can be maintained throughout several xenograft passages (**MANUSCRIPT I**). One exception is the xenograft passage GBM_CPH029p7 and thereof derived neurosphere cultures that seemed to be deprived of EGFRvIII expression whereas previous and latter xenograft passages were positive for this marker (**MANUSCRIPT I,** Fig. 3A and 3C). It has previously been reported that approximately 50% of the GBM cells in a tumor sample were EGFRvIII positive as assessed by flowcytometric analyses[24]. Vital *et al*. have suggested that intratumoral heterogeneity might be a result of genetic changes occurring throughout tumor development. As such, early changes would be present in the majority of tumor cells, while later changes only would be present in a subset of tumor cells[53]. It could as such be speculated that the EGFRvIII mutation is a fairly late event during tumor progression, thus leading to the existence of different bCSC populations within the same tumor. I.e. some bCSC and their progeny will be EGFRvIII positive and some bCSC and progeny will be EGFRvIII negative and incapable of regenerate that particular property when transplanted as xenograft onto mice. This scenario could explain why the xenograft passage 7 was the only passage derived from patient tumor GBM_CPH029 lacking EGFRvIII expression. In addition to maintenance of EGFR and EGFRvIII expression, the expression of the Notch-1 receptor was also maintained throughout *in vivo* passaging as displayed in Figure 12.

When we initially established the xenograft tumors *in vitro*, we did parallel cultures in serum-free neurobasal (NB + additives) and serum-containing (DMEM+10% FCS) culture media respectively. However, almost all of the serum-cultured cells (25 out of 27) failed in supporting the growth of the GBM cells, and were excluded as they either were very slow growing or expressed mouse actin and thus most likely were contaminated by normal mouse cells (data not shown). In contrast, more than 50% (16 out of 27) of xenografts established in NB-media were successfully established as neurosphere cultures and free of mouse actin (Table 1 above, **MANUSCRIPT I**, Fig. 5B and data not shown). The difference in the number of established NB-cultures between the above statement and Table 1 in **MANUSCRIPT I** is due to the fact that more than one xenograft passage from each of the five patient tumors was established as NB-cultures (except from patient tumor GBM_CPH017, see Table 1 above). We, as such, concluded that establishment of GBM *in vitro* cultures during serum-free culture conditions improve the success rate as compared to serum-containing cultures and maintain important patient tumor hallmarks.

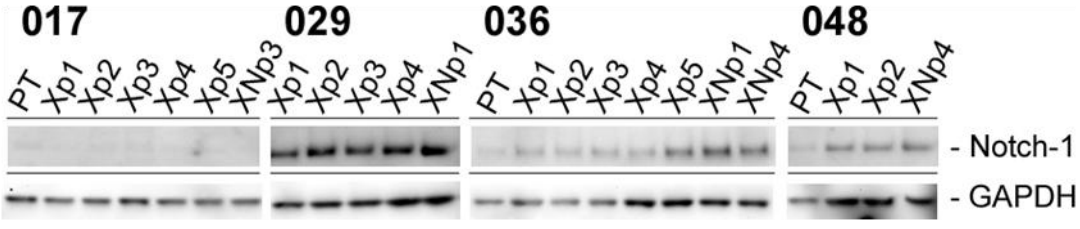


Figure 12: Expression of Notch-1 is maintained from patient tumor throughout xenograft passaging. Basal protein expression detected by western blotting of the Notch-1 receptor in patient tumor tissue and thereof derived different xenograft passages. 017: GBM_CPH017, 029: GBM_CPH029, 036: GBM_CPH036, 048: GBM_CPH048. The Notch-1 expression in the GBM_CPH047 patient tumor and thereof derived xenograft passages has not yet been determined. PT: Patient tumor, XpX: xenograft passage X, XNpX: xenograft passage X after the tumor has been passaged over nude rats to remove mouse hepatitis. GAPDH was used a loading control. Notice that the protein levels between the different tumors cannot be compared as they are not run on the same blot.

8.1.2 Characterization of GBM neurosphere cultures

In our model we used a xenograft intermediate station between the patient tumor and establishment of the neurosphere cultures, whereas others have established neurosphere cultures directly from patient tumor tissue[3,56]. However, both approaches seem to be an advanced model compared to serum containing cultures as they 1) improved the success rate as discussed above and 2) show a better resemblance to the patient counterpart with respect to genomic alterations, gene expression[3,56] and tumor specific markers as we demonstrated that the expression of EGFR/EGFRvIII and Notch-1 were maintained from *in vivo* xenografts throughout several passages of *in vitro* culturing (**MANUSCRIPT I**, Fig. 6 and **MANUSCRIPT III**, Fig. 2 and Figure 13 below). Moreover, by using a broader panel of patient tumors as the basis for experimental cell cultures it is possible to achieve a more authentic overall picture of the human disease, as all GBM sub-types are more likely to be covered by the model as compared to the traditional serum cultured commercial cell lines. It should however be held in mind that although patient tumor sub-types can be reflected in corresponding xenografts, sub-typing of *in vitro* cultures is not yet confirmed comparable with patient tumor sub-types[1].

As outlined in section 2.3, the neurosphere culture system supports growth of stem cell-like GBM cells, bCSC, that are believed to play a pivotal role in tumor initiation, progression, angiogenesis, treatment resistance and relapse. And as bCSC are believed to harbor NSC-like potential, the neurosphere cultures offer insight to the mechanisms behind the maintenance of bCSC. In support, the neurosphere cultures established in the present project could be characterized as having NSC-like potential. Besides the neurosphere cultures presented in **MANUSCRIPT III**, Fig. 1, we have also verified that cultures established from the NGBM_CPH047p2 xenografts exhibit NSC-like characteristics as they to formed neurospheres in primary culture and showed self-renewing and multipotent capacity. Neurosphere cultures established from NGBM_CPH017 tumors have not yet been attempted verified this way, although they grow as neurospheres in NB-media and form orthotopic tumors when injected intracranially into immunodeficient mice (data not shown).

It is important to mention that the neurosphere cultures are not purely composed of bCSC but rather a mix of more or less differentiated GBM cells[72,115]. Although many have reported that it is feasible to sort and isolate the bCSC population, there are continuous dispute about how to identify the bCSC. The NSC marker CD133 is to some extent still the golden standard when identifying and sorting a GBM stem cell-like cell population both from tumor tissue and cultures[14,17,26,71]. However

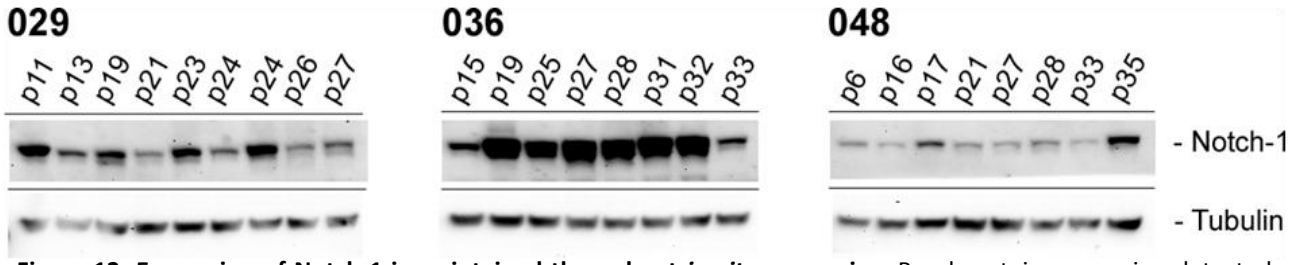


Figure 13: Expression of Notch-1 is maintained throughout *in vitro* passaging. Basal protein expression detected by western blotting of the Notch-1 receptor in different passages of patient derived neurosphere cultures. 029: GBM_CPH029p7, 036: NGBM_CPH036p7, NGBM_CPH048p6 (pX following the patient tumor name corresponds to the mouse passages from which the culture was derived). pX above the blots corresponds to the neurosphere passage. GAPDH was used a loading control. Notice that the protein levels between the different tumors cannot be compared as they are not run on the same blot.

reports on CD133 negative cells forming tumors[121,123] that furthermore gives rise to CD133 positive cells[122] raise question about the reliability for this marker as a marker for the self-renewing and tumor-forming GBM cell population[274]. The same accounts for using side-population sorting, that is based on the differential efflux of the Hoechst 33342 dye by the ABC (ATP-binding Cassette, ABCG2 in humans) multidrug transporter expressed by a minor population of GBM cells believed to be bCSC[275-277], as also ABCG2 negative cells has been shown to exhibit tumorigenic potential[276]. Moreover, the side-population has recently been shown to be stroma derived and primarily composed of endothelial cells, and thus not stem cells or cancer cells[278]. It has, in addition, been demonstrated by flow cytometry that the CD133 population is not comparable with the side-population[279]. The Nestin promoter has been used as a driver in genetically modified models as described in section 2.2.2, and Nestin has also been reported expressed in other cancers such as pancreatic, breast, ovarian, thyroid and prostate cancer[280] and has furthermore been suggested as a therapeutic target in malignant melanoma[281]. Nestin is also the stem cell marker we have had most success with detecting at the protein level (data not shown). Due to the uncertainties of specific bCSC markers, functional assays have gained increasing attention when identifying bCSC. As described in section 2.2.1 and 2.3.2 and as demonstrated in **MANUSCRIPT II, III** and **IV**, bCSC can be identified by means of their the stem cell characteristics, such as sphere formation, self-renewal and multipotency along side with the tumorigenic potential, with the latter being the clinically most important feature. And perhaps, the need for sorting and analyzing the bCSC population on its own might be more important when studying specific mechanisms within this cell population. In the case of modeling GBM, the neurosphere culture as a whole might be more relevant as it, at least to some extent, mimics the heterogeneity seen in the human tumor tissue. Either way, it has been shown that both a presumably isolated bCSC population[17] as well as the unsorted neurosphere culture are able to recapitulate the heterogenicity and characteristics exhibited by the patient tumor of origin (**MANUSCRIPT I** and **IV** and[282]) and thus fulfill the cancer stem cell hypothesis. It can as such be concluded that when establishing human GBM cells *in vitro*, regardless of origin, it is crucial that the culturing conditions used support the growth and maintenance of bCSC in order to preserve the geno- and pheno-type of the original patient tumor. And even with the lack of a specific molecular profile for the bCSC, it is most likely that targeting this cell population will lead to improved therapy and hence better prognosis for GBM patients[107,114,115]

8.1.3 Going back in vivo – can differences in the cultures explain differences in growth pattern?

During the time-frame of this thesis project, at least one neurosphere culture derived from each of the five original patient tumors has been established both as subcutaneous and orthotopic tumors in immunodeficient mice, and has as such been verified to retain the tumorigenic potential (**MANUSCRIPTS I** and **IV**, and unpublished data). As described in section 2.2.2, although more laborious, an orthotopic tumor model is believed to be a more accurate model than a subcutaneous model, as the micro environment, in this case the brain parenchyma and stroma, better mimics the original tumor micro environment with regard to vascularization, available growth factors, stromal-, and other non-malignant cells making up tumor cell niches[92]. Furthermore, GBM cells established as orthotopic xenografts better resemble the human counterpart than subcutaneous xenografts[91]. The three neurospheres cultures used for orthotopic tumor formation in the present study, the

GBM_CPH029p7 (029), NGBM_CPH036p7 (036) and NGBM_CPH048p6 (048) cultures (**MANUSCRIPT IV**), all formed tumors with unique GBM characteristics, such as necrosis and micro vascular proliferation but also atypical mitosis and invasion was detected (**MANUSCRIPT IV**, Fig. 2). However, the *in vitro* growth could not predict how the tumors grew *in vivo*. *In vitro*, the 029 and 036 cultures were fast growing, formed large dense spheres and had to be passaged at least once a week, while the 048 culture formed less densely packed spheres and was only passaged once every two weeks (data not shown). However, intracranial tumor formation revealed a very different picture were mice injected with 048 cells survived maximum 7 weeks, mice injected with 029 cells maximum 19 weeks and 036 mice survived as long as 38 weeks or 9,5 months. The histological appearance of the tumors was also very different. The 048 tumors consisted of a large, in general uniform and well bordered, central tumor with occasional small islands of tumor tissue located elsewhere. The 036 tumors tended to be located in, and/or in proximity to, the ventricles and were more infiltrative. In addition, some of the 036 tumors had large, although non-malignant looking, vessels. Finally the 029 tumors showed the most pronounced infiltrative behavior and were also located in, and/or in close proximity, to the ventricles. High expression of EGFR has been linked to a poor prognosis[46] and as the 048 showed a higher level of EGFR (**MANUSCRIPT I**, Fig. 3 and 6, and Appendix 1) compared to the 036 and 029, most likely do to *EGFR* amplification (**MANUSCRIPT I**, Table 3 and 5), this might at least partly explain why the 048 tumors grew much faster than the 029 and especially 036 tumors *in vivo*. However, it does not explain the discrepancy between the growth *in vitro* and *in vivo* respectively. This might be explained by the observation that the 029 and 036 tumors grew more infiltrative and it could be speculated that infiltrative growth is a slower process due to degradation of tissue barriers as also discussed in **MANUSCRIPT IV**. It could also be speculated that the difference in growth rate between *in vivo* and *in vitro* conditions is due to the difference in micro environment. The 029 and especially the 036 tumors were located in and around the ventricles, a phenomenon also observed by others[97], and perhaps these cultures more closely resemble cells within these areas, and therefore home to these locations. Normal NSC are known to be naturally present in the sub-ventricular areas and perhaps the cells within the 029 and 036 cultures are more bCSC like, than cells in the 048 culture. However, when evaluating the expression of the stem cell marker Nestin (**MANUSCRIPT III**, Fig. 1), this does not seems to be the case, as there is no major difference in Nestin expression between the cultures. Instead, maybe the bCSC population in 029 and 036 represents a different sub-type than in the 048. Indeed, it has been reported, that different bCSC cultures can be obtained from the core and the periphery of the tumor respectively, although derived from a common ancestor[283]. It could be speculated that this is a result of early and late genomic alterations[53] as described above. In addition, a three dimensional GBM model has been proposed were the hypoxic gradient in the tumor defines the molecular and phenotypic characteristics of the tumor cells with the more immature GBM cells being present near the hypoxic core of the tumor[284]. In line with this, it has been demonstrated that stem cell features are promoted by hypoxia[129,131]. It must, however, be considered that different hypoxic areas can be present within the same tumor as evident by the observation of different necrotic areas when evaluating the H&E sections in **MANUSCRIPT IV**, data not shown. Taken together, in this study, the tumor specimen available from each patient tumor might have been from different locations within the tumor and thus could represent different bCSC populations. This is further supported when

examining the expression of neurogenic genes in the untreated cultures (Appendix 3). Here the expression pattern of 029 and 036 cultures were more alike whereas the 048 culture almost showed an opposite expression pattern, indicating that the different cultures represent two distinct types of GBM, both of which contain cells with tumor initiating capacity. The difference between the examined cultures might be a result of the tumors belonging to different GBM tumor sub-types and thus originate from different cell types as has been suggested by Woehrer *et al.*[2]. However, this is still speculative and additional analyses are required to clarify this.

8.1.4 Visualizing the orthotopic GBM model

In order to be able to use an orthotopic tumor model for pre-clinical research it has to be possible to monitor tumor growth and response to treatment while the animals are still alive. As GBM is a neurological condition, the symptoms are as diverse as the location of the tumor in the brain as outlined in section 2.1. However, apart from other brain tumors and brain metastases, these symptoms can also be caused by none malignant conditions such as blood vessel malformation, infection, hemorrhage, infarction, multiple sclerosis etc.[285]. Different biomarkers implicating a malignant condition have been identified in the blood plasma, such as the inflammation marker FTL (representative proteins-ferretin light chain)[286]. Also biomarkers directly found to be altered in GBM patients compared to healthy controls, such as S100A9 (member of the Ca^{2+} signaling cascade) and CNDP1 (carnosine dipeptidase-1)[286] and biomarkers known from the literature to be closely associated with high-grade gliomas and GBM, such as YLK-40[287] and GFAP[288], have been suggested as potential plasma biomarkers. However, plasma biomarkers are at present not used as a diagnostic tool.

To confirm the presence of an intracranial tumor, different real time imaging techniques can be utilized. For this Magnetic Resonance Imaging (MRI)-scanning is currently the imaging modality of choice[289]. In a T1-weigthed MRI image with the addition of gadolinium as contrast the tumor is typically seen as a contrast enhanced ring that is believed to represent densely packed neoplastic cells with abnormal vessel structure and a central dark core that represents necrosis, while in a T2-weighted image (does not rely on contrast) the tumor area appears as a bright zone representing edema. However, several studies have shown that the area of enhancement on MRI is not an exact representation of the actual tumor border as infiltrative tumor cells can be found beyond a 2 cm. margin[28,290]. Another pitfall is that the contrast enhancement is not by definition brain tumor tissue as other lesions such as abscesses, metastases and tumor-like demyelating lesions in a person with tumefactive multiple sclerosis[291] also result in enhancement [292]. Thus MRI gives the suspicion of a brain tumor, however, it has to be confirmed and graded by pathological analysis of a stereotactic biopsy or a craniotomy. As such, neuropathologists give the final diagnosis of GBM by H&E staining and further determine if it is of primary or secondary origin by IHC analyses of EGFR, p53, IHD1 etc.[6]. Furthermore, MRI-scans do not visualize the actual tumor but the blood flow and fluid in and around the tumor, representing edema. This can lead to false-positive results when evaluating response to treatment and it has been suggested that the tumor shrinkage seen after bevacizumab treatment is caused by normalization of the blood vessels[293] and it is as such the diminished edema rather than tumor shrinkage that is visualized, a phenomena also referred to as pseudo-response[294].

Positron Emission Tomography (PET)-scanning is a way to non-invasively measure metabolic processes *in vivo*. By injecting a radioactive tracer that specifically binds to or is taken up by the tumor cells, the actual tumor cells can be visualized. A PET scan is often combined with a CT-scan as no anatomic information is visible in the PET image (Figure 14A). Moreover, CT-scanning by itself is not very usable for visualizing brain tumors as it has low resolution in soft tissue such as the brain (Figure 14B). However, when a CT image is combined with a PET image where the tumor cells emit a brighter signal than the surrounding non-malignant tissue, it is possible to anatomically locate the tumor bulk (Figure 14C). Different tracers can be utilized for PET: FDG (2-deoxy-2-^{18}F-flouro-D-glucose) is a glucose analogue, which also is absorbed in normal brain tissue and inflammatory tissue and as such gives a high background signal. Due to the low specificity of FDG in the brain, this tracer is not routinely used for brain tumor imaging[295]. FLT (^{18}F-3'-fluoro-3'-deoxy-L-thymidine) is a thymidine analogue, that, because of its inability to cross the blood-brain barrier (BBB) is dependent on leaky vessels[296], and again this could lead to pseudo response when evaluating e.g. anti-angiogenic treatment as described above. For the present project we used the tyrosine analogue FET (*O*-(2-[F]flouroethyl)-L-tyrosine) tracer as it has shown great specificity when detecting brain tumors in patients[297,298]. FET enters the cancer cells by uptake mediated by amino acid transporters but is not incorporated into proteins. It is believed that the low background in non-malignant tissue is mainly due to selective uptake into the cancer cells by the L-type amino acid carrier system (reviewed in Langen *et al*. (2006)[299]). In consensus, we found the FET tracer to be highly specific for detecting intracranial tumors from our neurosphere GBM cells and tumor formation was confirmed by histological analyses in all mice displaying an intracranial FET signal (**MANUSCRIPT IV** and data not shown). However, the lack of a FET signal was not synonymous with no tumor, as mice that were euthanized without visible tumor as detected by FET, had detectable tumor tissue by H&E staining. These tumors were nevertheless rather small. E.g. one mouse was euthanized at day 8 after injection, with no detectable intracranial FET signal, although a small lesion of established tumor cells could be observed in the H&E section (Figure 15), which indicates that the tumor has to be above a certain size in order to be detected by CT/FET-PET.

Of more practical orientation we did experience some difficulties when injecting the tracer *intravenously* (*i.v.*) as the anesthetic gas (isoflouran) initially used turned out to be vaso-contracting, a phenomenon also experienced by colleagues (Personal communication with technical staff at the Department of Experimental Medicine, University of Copenhagen). As a solution we were advised to switch to hypnorm-medizolam as it is known to be vaso-dilating. However, SCID[h]-mice tolerate repeatedly sedation poorly, and approximately two mice per group in the study presented in **MANUSCRIPT IV** were euthanized due to symptoms that most likely were not caused by

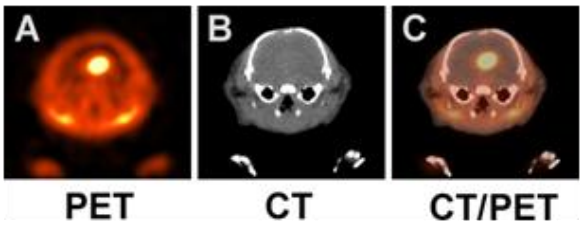


Figure 14: Live imaging of intracranial tumor tissue in the orthotopic GBM model. A) FET-PET image, B) CT image, C) CT image combined with FET-PET image. The images were obtained by injecting *O*-(2-[F]flouroethyl)-L-tyrosine (FET) and recording the signal after 20 minutes under hypnorm-midazolam anesthesia.

[h] spontaneous mutant T & B cell deficient mice

tumor growth, although this is merely speculation as tumor tissue could be detected in the brain by histology afterwards. Nonetheless, these mice were not included in the survival data presented in **MANUSCRIPT IV** (Fig. 2A, Fig. 4B-D and Supplementary Fig. S3). An alternative when performing frequent PET-scannings could be to use NMRI[i]-nu mice that presumably tolerate the repeated sedation better (unpublished data). In addition, when analyzing the brains by H&E staining, from mice euthanized solely due to tumor related signs, we found that histological tumor size not was proportional with survival as some mice had small tumors, but severe weight loss. These small tumors were often located in the ventricle system (**MANUSRIPT IV**, Supplementary Fig. S3) and the symptoms as such could be a results of increased intra ventricular pressure (hydrocephalus)[300].

Taken together, the *in vivo*/*in vitro* model utilized as the basis in this project, where neurosphere cultures were established from patient derived GBM xenografts, represents a reliable GBM model as important tumor characteristics and bCSC features are maintained throughout the model. Furthermore, the established neurosphere cultures could be injected orthotopically into the brains of immunedeficient mice where they formed intracranial tumors verifying the tumorigenic potential of the cells. Thus, with some adjustments regarding imaging, this model could serve as an invaluable tool when examining the functional role of promising GBM specific targets and when testing the effect of potential new anti-GBM therapies on tumor initiation and growth. In this regard, preliminary data have demonstrated that it is possible to detect luciferase transfected 048p6 cells by bio-luminescence at week one after intracranial injection (Appendix 4).

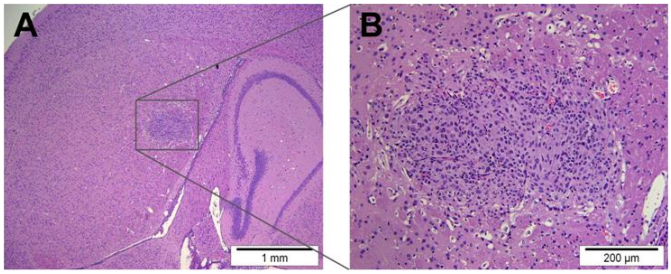


Figure 15: Early detection of tumor cells in the mouse brain. H&E staining of brain sections from a mouse euthanized eight days after injection with 048 cells at two different magnifications. No PET signal could be observed in this mouse (data not shown).

8.2 Studying the role of EGFR and Notch signaling in bCSC

The above discussed *in vivo*/*in vitro* model has made out the backbone when we explored the significance of the EGFR and Notch signaling pathways, known to be important for the maintenance and features of normal NSC and also believed to play a role in bCSC and GBM malignancy.

Although anti-EGFR treatment for other cancer types, such as non-small-cell lung cancer, head and neck squamous cell carcinoma and metastatic colorectal carcinoma (reviewed in Nedergaard *et al.* (2012)[161]), have proved valuable in the clinic, successful targeting of the EGFR pathway in GBM is still in large unachieved. One example is the lack of effect from cetuximab on glioma cell growth *in vitro*[201] and on the response rate and survival of recurrent GBM patients when added to an irrinotican and bevacizumab treatment regimen[197]. However, as EGFR has been implicated in many cancer types including GBM, where amplification of the *EGFR* gene often is accompanied by the mutant variant EGFRvIII (**MANUSCRIPT I** and[55,301]), and moreover has been associated with the

[i] spontaneous mutant t-cell deficient mice

immature cells of the nervous system and bCSC, targeting of EGFR might still be feasible under innovative setups as will be discussed below. The Notch pathway is, as EGFR, known to be important in the normal NSC population and is likewise deregulated in GBM. Mutations of the Notch receptors have been found in other cancer types[259], however, Notch mutations are not common in GBM and exogenous activation of the receptor alone has proven inefficient for generating lesions in the SVZ[230]. Nevertheless, an increasing interest for the role of this pathway in GBM has emerged in the latter years.

8.2.1 Expression of EGFR and Notch in the in vivo/in vitro GBM model

As described in section 2.4.2, EGFR is over expressed in 50-60% of GBM and mutated in 40-50% of which EGFRvIII is the most common. This is in line with our observations as 44%-47% of the patient tumors we have established in our *in vivo*/*in vitro* model (**MANUSCRIPT I**, Table 1) were positive for EGFR as assessed by WB and IHC respectively. In contrast only four (five, when counting in the GBM_CPH047 tumor, that was EGFRvIII negative in the patient tumor, but positive in the following xenografts and neurosphere cultures) out of 36 patient tumors were positive for the EGFRvIII. This could be an inconsistency caused be the heterogenic nature of GBM as discussed in **MANUSCRIPT I** and therefore EGFRvIII might be absent in the tumor biopsy available for the project although expressed in other areas of the tumor. However, tumors positive for EGFRvIII were also positive for EGFR expression as assessed either by WB or IHC which supports previous reports on EGFRvIII predominantly being expressed in tumors with EGFR amplification[176]. To summarize: of the five patient tumors established as neurosphere cultures and explored in the present project, we found that the 017p4 and 036p7 were possibly EGFR wild type, the 029p7 and 048p6 were possibly amplified for EGFR, while the 047p2 and 047p3 cultures were found to be EGFRvIII positive as well as possibly EGFR amplified. It should be noticed that previous and later *in vivo* passages of GBM_CPH029 were found to be EGFRvIII positive (**MANUSCRIPT I** and discussed in section 8.1.1).

With regard to expression of Notch pathway components we initially categorized the xenografts and thereof derived neurosphere cultures based on the expression of the Notch-1 receptor, the downstream target Hes-1 and the overall response to Notch inhibition by DAPT treatment (**MANUSCRIPT III**). This resulted in three cultures (029p7, 036p7 and 047p2) being characterized as having high endogenous Notch-1 expression and activation, while two cultures (017p4 and 048p6) were characterized as having low expression and activation. Others have reported difference in the expression of Notch and Hes between GBM samples[105,302] and low activation of the Notch pathway has been linked to progressive gliomas and thus secondary GBM[303], while others have shown that Notch-1 expression increases with increasing glioma grade[211]. In contrast, increased expression of Notch pathway components have been reported in low grade gliomas compared to GBM[208]. However, the GBM tumor tissue used in this project has not been compared to lower grades of gliomas or normal brain tissue and the level of Notch expression can as such not be categorized as high or low compared to these tissue types. Moreover, the present GBM samples have all been diagnosed as primary GBM (**MANUSCRIPT I**) and as the study by Verhaak *et al*. (outlined in section 2.1.1) demonstrate, the level of Notch expression can be associated to a specific GBM sub-type[1], which will be discussed below.

When evaluating the gene expression of selected glioma and Notch components (Appendix 1 and 2) from the gene expression analysis performed in **MANUSCRIPT IV**, it becomes evident that the neurosphere cultures examined indeed are different. (Notice that the 017p4 culture is not included in this analysis and that the 047p3 culture for simplicity reasons is displayed only in the cluster analysis in the appendix, while absent in **MANUSCRIPT IV**). As shown in Appendix 1, the 048p6 culture displayed increased expression of EGFR as compared to the other three cultures, verifying this culture as having the highest degree of *EGFR* amplification (**MANUSCRIPT I**). This culture also showed the highest level of the negative regulator PTEN, which might indicate a low signaling through the PI3-K/AKT pathway, however, the gene expression data is inconclusive in that regard as different PI3-K and AKT transcripts lack uniform up- or down-regulation compared to the other cultures (Appendix 1a). The 047p3 culture showed the lowest level of the EGFR ligands EGF and TGF-α which might be explained by the EGFRvIII status of this culture (**MANUSCRIPT I**) and thus the independence of an autocrine growth factor loop[164]. Furthermore, this culture showed increased expression of the AKT homolog 2 and 3 compared to the other three cultures, in line with studies suggesting that EGFRvIII has an increased signaling through the PI3-K/AKT pathway[173,175], as outlined in section 2.4.2. The 029p7 and the 036p7 showed the closest resemblance in this Glioma gene expression analysis (Appendix 1a). The same is the case when evaluating the expression of the Notch signaling pathway, except for some of the genes, e.g. 036p7 showed the highest expression of Dll-4 in the second cluster (Appendix 2a). Interestingly, as described in section 8.1.3, the 036 orthotopic tumors displayed a higher degree of vessels compared to tumors from the other cell cultures (**MANUSCRIPT IV**), which we also have observed in the subcutaneous tumors (data not shown) and Dll-4-Notch signaling has recently been linked to large blood vessels[272] and increased tumor angiogenesis[273]. The 048p6 culture almost displayed an opposite Notch signature profile when compared to the 029p7 and 036p7 cultures as the Notch-1, 2- and -3 receptors, the ligands Dll-1, Jagged-1 and -2, and the downstream targets Hes-1, Hey-1 (**MANUSCRIPT IV**, Fig. 1) as well as the transcription factor CSL (RBPJ, Appendix 2a) all were expressed at a lower level in 048p6, which might explain the difference in response to Notch inhibition observed in **MANUSCRIPT III** (discussed below). The 047p3 culture mostly resembles the 029p7 and 036p7 (Appendix 2a), although, with regard to the third cluster, it in general displayed the lowest expression compared to the other three cultures (e.g. Hes-1 and Jagged-1 and -2). Surprisingly 047p3 showed same expression level of Notch-1 (and Notch-3) as 048p6, which is in contrast to our findings in **MANUSCRIPT III,** Fig 5B, where we characterize 047p2 as having high Notch-1 expression. However, the results are from two different 047 xenograft passages (p3 and p2 respectively) and the characterization in **MANUSCRIPT III** was based on protein level, while the cluster analysis was based on mRNA level, and the discrepancy might as such be due to post-transcriptional regulation of the Notch-1 receptor. E.g. the RNA-binding protein Musashi-1 has been shown to regulate Notch expression in the embryonal brain tumor medulloblastoma as well as in GBM[304,305] and, furthermore, several GBM-specific micro-RNAs (miRs) have identified to be involved in the Notch pathway of which e.g. the miR-137 inhibited Notch-1[306]. Nevertheless, it is evident from the gene expression analysis of Notch pathway components, that the four cultures examined, displayed different Notch signatures with the 029p7 and 036p7 being most alike.

When comparing overall gene expression analysis with the sub-typing studies described in section 2.1.1, it becomes obvious that no good correlation of the neurosphere cultures analyzed in this project and the different sub-types suggested by Verhaak *et al*.[1], Brennan *et al*.[48] and Phillips *et al*.[22] can be made. As an example, the classical sub-type in the Verhaak study and the EGFR core in the Brennan study are characterized by increased expression of EGFR, which fits with the 048p6 cultures (**MANUSCRIPT I**). However, Verhaak and Brennan also ascribe up regulated Notch signaling to the classical sub-type and EGFR core respectively, which do not match the 048p6 profile (**MANUSCRIPT III** and **IV**). In contrast, Phillips assigned increased EGFR expression to the Proliferative and Mesenchymal sub-type, while they did not find altered expression of the Notch receptors in any of the sub-types. However, these sub-type classifications were based on several hundred glioma samples of both grade III and IV origin. Moreover, the expression characteristics were not exclusive for the individual sub-type, as well as overlap between the sub-types in the different studies could be observed (reviewed in Woehrer *et al*. (2013)[2] and Huse *et al*. (2011)[307]). As such, the expression profiles established for GBM cells grown as neurosphere cultures in the present project are not immediately comparable with previously published sub-type profiles, but it must be considered that further sub-classification within the sub-types could be present. It should furthermore be held in mind that the three studies described in section 2.1.1 only represent a subset of sub-classification studies all with variations in the sub-typing, although the features distinguishing between a mesenchymal and a proneural sub-type in general were consistent (reviewed in Huse *et al*. (2011)[307]). Regardless, establishing GBM cells as neurosphere cultures, still must be considered a superior model as compared to serum-containing cultures, as discussed above, as it better maintains patient tumor characteristics as well and GBM hallmarks and as such mimic the patient disease better[3].

8.2.2 Are there similarities between the role of EGFR and Notch in the in vitro model?

It is important to emphasize that the experiments and results in **MANUSCRIPT II** and **III** cannot be directly compared as 1) the GBM neurosphere cultures analyzed display different molecular expression profiles as described above and 2) the setup varied a bit with regard to the cellular assays utilized in the two studies (e.g. the sub-sphere and the soft-agar assays), which will be outlined below. With that in mind, a comparison of the results in the two studies will nevertheless be performed in the following in an attempt to decipher the similarities and/or differences between the functionality of EGFR and Notch, respectively, in the neurosphere cultures and thus the bCSC population. An overview of the results obtained in **MANUSCRIPT II** and **III** is displayed in Table 2.

One might speculate that only tumors that show increased expression and activation of a specific pathway should be stratified to therapy targeting this pathway as suggested with regard to the sub-type classification. In consensus, it has been suggested that amplification of EGFR improves response to EGFR inhibition by TKIs, if it is combined with low levels of activated AKT [187,188,308] and that EGFRvIII positive tumors are more sensitive to cetuximab treatment, than EGFRvIII negative tumors[202]. Furthermore, we have shown that only neurosphere cultures characterized as having high endogenous Notch pathway activation were sensitive towards anti-Notch treatment by GSI (**MANUSCRIPT III**). To explore the functional role of EGFR/EGFRvIII and Notch signaling in bCSC we therefore utilized the 047p3 neurosphere culture demonstrated to express

Table 2. Overview of the *in vitro* results obtained in MANUSCRIPT II and III

Treatment	Primary sphere	Sub-sphere		Differen-tiation	Soft-agar		Viability
		Naïve	Pre-treated		Naïve	Pre-treated	
AG1478	⇩	N.A.	⇩	⇧	⇩	N.A.	⇩
DAPT	⇩	-	-	-	-	⇩	-
ICN-1	N.A.	⇧	⇧	⇩	⇧	N.A.	N.A.
RA	N.A.	N.A.	⇧	⇧	⇩	N.A.	N.A.
Serum	N.A.	N.A.	⇩	⇧	-	N.A.	N.A.

⇩ : Inhibitory effect from treatment in the respective assay, compared to the control
\- : No effect or no consistent effect from treatment in the respective assay, compared to the control
⇧ : Promoting effect from treatment in the respective assay, compared to the control
N.A. : Not analyzed

EGFR/EGFRvIII (**MANUSCRIPT II**) and the 029p7, 036p7 and 048p6 cultures, representing high and low Notch-1 expressing neurosphere cultures, respectively (**MANUSCRIPT III** and **IV**).

Even before the first reports on bCSC, it was demonstrated that EGFR knock-out in the glioma cell line U87MG led to differentiation and reduced growth and colony forming potential[309]. In addition, NSC are known to proliferate in response to EGFR ligands such as EGF, bFGF, and withdrawal of growth factors has been shown to induce differentiation[60,62]. As increased grade of anaplasia or de-differentiation is linked to increased aggressiveness, it could be speculated that EGFR signaling plays a role in upholding the immature state of bCSC important for maintaining the malignancy of the tumor. Likewise is it known that active Notch signaling is important for maintaining the balance between the undifferentiated NSC population and its differentiated progeny. Thus, as NSC are a possible origin for bCSC, and as Notch signaling has been suggested to drive expression of the NSC marker Nestin[230], it is not farfetched that Notch signaling also plays a role in the maintenance of bCSC, as also has been suggested by others[26,267,268]. Taken together, both EGFR and Notch signaling are proposed a role in bCSC maintenance, the cancer cell population believed to be a promising target in novel anti-GBM treatment.

The sphere forming potential is a well established NSC/bCSC characteristic and sphere formation has furthermore been correlated to clinical outcome of high-grade gliomas[310]. As such, we have interpreted the number of neurospheres formed when the primary culture was established in serum-free media as a pseudo quantification of the bCSC population present in the tumor tissue from which the culture was established. This way we tested if inhibition of EGFR/EGFRvIII by the TKI AG1478 or Notch inhibition by the GSI DAPT had an effect on the primary sphere formation and thus on the supposed bCSC population. Indeed, we found that AG1478 reduced the number of primary spheres in all the cultures analyzed this way (047p2 is displayed in **MANUSCRIPT II**, Fig. 4D. Data not shown for 029p5[j] and 048p7. 017pX and 036pX were not analyzed), while Notch inhibition only affected the sphere forming capacity in the high Notch-1 expressing cultures (029p5, 036p8, 036p15 and 047p2, **MANUSCRIPT III**, Fig. 5A). Thus, these results indicate that primary

[j] The NGBM_CPH029p5 xenograft was positive for EGFRvIII in contrast to GBM_CPH029p7

sphere formation to some degree is dependent on EGFR and Notch signaling and that both EGFR/EGFRvIII and Notch inhibition affect the bCSC population in the tumor. It could be speculated if AG1478 also would have hampered primary sphere formation in 017pX and 036px as these were categorized as having normal EGFR expression (section 8.2.1) in line with the above discussion regarding that sensitivity to a pathway inhibitor requires abnormal signaling through that specific pathway, however, this remains to be explored. The results from the primary sphere formation is in line with others showing that primary glioma (and NSC) sphere formation was inhibited by GSI[267] and reports showing that the number of primary spheres was significantly increased along with an increase in sphere size when GBM cells were established in serum-free media in presence of EGF and bFGF compared to establishment in the absence of growth factors[108]. It could be argued that the lack of growth factors would induce differentiation of the GBM cells as described in section 2.1.1, however, the authors did not find increased differentiation in the cultures that were established deprived of growth factors[108]. Moreover, differentiation studies are mainly performed on already established cultures[62,72], and later passage cultures most likely are composed of a different cellular makeup compared to primary cultures that also would affect the bCSC potential in the cultures (discussed in **MANUSCRIPT II** and **III**).

In later *in vitro* passages we found that the 047p3 culture formed fewer spheres when subjected to EGFR inhibition (**MANUSCRIPT II**, Fig. 4C) in line with results from Kelly *et al.*[108], while no effect of Notch inhibition was observed in 029p7, 036p7 or 048p6, regardless of Notch signature (**MANUSCRIPT III**, Fig. 5 C and D) and in contrast to what previously has been reported[267]. The same was the case when evaluating the expression of differentiation markers after treatment with either AG1478 or DAPT. Here we found that AG1478 induced differentiation (**MANUSCRIPT II**, Fig. 3E), while no consistent effect on the differentiation markers could be detected upon DAPT treatment (**MANUSCRIPT III**, Fig. 5E and F). Also when testing the effect on neurosphere cell viability, we found that AG1478 reduced the quantity of viable 047p3 cells (**MANUSCRIPT II**, Fig. 3B) in line with a study by Soeda and colleagues[282], while no consistent results were obtained upon treatment with different concentrations of DAPT in the 029p7, 036p7 and 048p6 (data not shown), which is in contrast to other studies[26]. AG1478 was evaluated after 12 days, whereas the DAPT treatment only was sustained for 3 days and the viability assays can as such not be directly compared as also emphasized above. Nonetherless, after three days of DAPT treatment we did see an effect on the cell cycle distribution (**MANUSCRIPT III**, Fig. 3A and 6B), and the time frame might thus, not have been enough to manifest as a decrease in the amount of viable cells. In fact, in a recent Master´s thesis project from our laboratory it was demonstrated that the viability of 036p6 and 047p3 neurosphere cells was decreased after 14 days of DAPT treatment, although the EGFR inhibitor gefitinib seemed to inhibit the viability to a higher degree than DAPT[311].

It could be hypothesized that the different results from the functional stem cell assays discussed above, is a result of the different means by which EGFR and Notch signaling is activated. EGFR ligands are present in the media, and EGFR signaling can as such can be initiated by normal means in our cell cultures. Furthermore, as described above, certain requirements have been suggested to be important for obtaining an effect from TKIs such as EGFR amplification and mutations, a signature the 047p3 culture fulfill. Perhaps an EGFR wild type GBM neurosphere culture would not have been affected to the same degree. This does, however, not seem to be the case as the viability

of the 036p6 culture, characterized as possibly EGFR wild type, was affected by TKI treatment, as described above[311]. Moreover, with regard to sub-sphere formation, the same master´s thesis project demonstrated that the sphere morphology of both the 036p6 and the 047p3 culture was affected by AG1478 and gefitinib[311] and Soeda *et al*. did not find increased sensitivity towards the same TKIs in EGFRvIII positive glioma neurosphere cultures when compared to EGFRvIII negative cultures[282]. As such, future studies needs to further compare the effect from EGFR inhibition on GBM neurosphere cultures with different EGFR/EGFRvIII expression. In contrast to EGFR activation, Notch signaling requires cell-cell interactions and is thus dependent on the presence of ligand expressing cells. As shown in Appendix 2a, ligands are expressed in the cultures (e.g. Dll-4 and Jagged-1), although at various levels and as such ligand activation of Notch signaling is plausible. Nevertheless, the ligand expressing cells in the culture can be different from the ligand expressing cells *in vivo* where the microenvironment is different, and the extracellular activation of the pathway might as such differ between *in vivo* and *in vitro* conditions (as discussed in **MANUSCRIPT III**), which might not be the case with EGFR. In addition, formation of an intracellular cell autonomous ligand-receptor complex, has been reported[312], and it could be speculated if activation of the Notch receptor in this complex requires γ-secreatse, as the receptor-ligand complex remains inside the cell and the γ-secretase is embedded in the plasma membrane[312]. Moreover, a noncanonical Notch signaling pathway independent of activation by γ-secretase has been suggested[313]. Thus, Notch signaling have alternatives to the canonical GSI sensitive pathway in order exert its downstream effects *in vitro*. These alternatives might be independent of γ-secretase activity and thus insensitive to GSI, with regard to the stem cell features. However, this is highly speculative and further studies are required to clarify this. On the other hand, maybe DAPT is not a potent Notch inhibitor, which has been demonstrated by others[314], and it could be hypothesized that the effect from DAPT treatment differs between different cell populations and/or functions. When we activated signaling downstream from the Notch-1 receptor by means of transfection with the intracellular Notch-1 receptor (ICN-1), we were able to obtain an effect on the stem cell characteristics in all cell cultures (Table 2 and **MANUSCRIPT III**, Fig. 6), verifying that active Notch signaling does play a role in the immature cell population in the neurosphere culture.

In order to investigate the role of EGFR/EGFRvIII and Notch signaling on the tumorigenic potential of the neurosphere cells, we used the pseudo tumorigenic soft-agar assay. Both EGFR/EGFRvIII inhibition by AG1478 treatment (**MANUSCRIPT II**, Fig. 4A) and Notch inhibition by DAPT treatment (**MANUSCRIPT III**, Fig. 4B) reduced the number clonogenic colonies (for an overview see Table 2). However, Notch inhibition only affected colony formation when the neurosphere cells had been pre-treated before casted in the semisolid agar with additional DAPT treatment, while no effect could be observed, when the cells were plated directly in the assay, which is in line with the study by Fan *et al*.[26], but differs from our results with EGFR/EGFRvIII inhibition. Again, the two studies cannot be directly compared, but it could be speculated that EGFR inhibition targets the neurosphere cells by different means than Notch inhibition. Perhaps EGFR inhibition directly inhibits the clonogenic proliferation of the colony forming cells together with every proliferating cell in the culture, while Notch inhibition merely differentiate the colony forming cells into proliferating progenitor cells that initially are able to form colonies, but lose that ability upon replating as also discussed in **MANUSRIPT III**. This speculation is in line with the inconsistent effect

on differentiation markers upon Notch inhibition observed in **MANUSCRIPT III,** fig. 5E and F and the established role of Notch as a regulator of the balance between the normal NSC and its more differentiated progeny as described in section 2.5.2, although further studies are required to clarify this.

When testing the effect of the differentiating agent RA, we found that although it down regulated the expression of EGFR/EGFRvIII, induced differentiation and inhibited the number of colonies formed in soft agar, it actually increased the number of spheres formed in the sub sphere assay, were EGFR inhibition decreased the number of sub-spheres (**MANUSCRIPT II**, Fig. 4A and B) (For overview, see Table 2). As a passing remark, RA treatment of GBM neurosphere cells has also been reported to down regulate the Notch pathway [315]. This result is in contrast to other reports showing that RA inhibited GBM neurosphere formation[315]. As described above, DAPT treatment also affected colony formation, but failed to hamper secondary sphere formation (**MANUSCRIPT III**, Fig. 4B and 5D). (It should again be emphasized that the assays cannot be directly compared). As discussed in the respective papers, the lack of inhibiting effect on sub-sphere formation from treatment with either RA or DAPT might be explained by these two treatments only partly differentiate the cells, and as such lead to the generation of faster proliferating progenitor cells. In **MANUSCRIPT II**, the treatment ceased upon seeding in the assay, while the treatment in **MANUSCRIPT III** was maintained throughout the experiment. This could explain why the number of spheres was increased in case of RA pretreatment, as it might have been fast proliferating progenitor cells without any inhibitory treatment, that were evaluated in the assay, while the continued DAPT treatment may have prevented this in **MANUSCRIPT III**. These results combined, suggest that the anti-proliferating effect of differentiating treatment of bCSC is reversible, and that it is as such crucial that either the treatment is continued, or that the supposedly generated progenitor cells are targeted by different means, such as conventional chemo therapy that targets fast proliferating cells, e.g. TMZ.

8.2.3 Functional role of Notch in the in vivo model

The results from the soft agar assay with DAPT treatment (**MANUSCRIPT III**, Fig. 4) made out the draft for the orthotopic *in vivo* studies of Notch function. It was our hypothesis that inhibition of Notch signaling targeted an undifferentiated clonogenic tumor initiating cell, possibly the bCSC, and that inhibition of this would hamper tumor growth, as has been demonstrated in the Fan study[26]. However, pretreatment of the neurosphere cells with DAPT before injection did not improve the survival of the mice (**MANUSCRIPT IV**, Fig. 3B-D), regardless of the Notch signature of the cells injected (029p7, 036p7 and 048p6). In fact, mice injected with DAPT treated 036p7 cells tended to survive longer than mice injected with the control treated cells. The lack of tumor growth inhibition could be explained by the recapitulation of Notch signaling, when the GSI treatment was withdrawn upon intracranial injection. This is supported by the Hes-1 expression which was not obviously down regulated in the DAPT tumors as compared to the controls, when the mice were euthanized (IHC data not shown[k]) which is in line with the results from Fan and co-workers. They, nevertheless, did obtain increased survival when the neurosphere cells were pretreated before

[k] It should be noticed that, with regard to the present project, the IHC staining for Hes-1 has not been fully optimized , but the preliminary results implicate that Hes-1 is expressed at equal levels between the DAPT and control tumors.

intracranial injection[26]. We did, however, find indications of increased differentiation as visualized by reduced Nestin expression, either focally or as a weaker staining throughout the tumor in general. In addition, we found an increased number of malignant appearing vessels in the tumors formed from pretreated 029p7 and 036p7 neurosphere cells (**MANUSCRIPT IV**, Fig. 4G-L, 4S-Y and Table 1). When taken the *in vitro* results into account (**MANUSCRIPT III**), the *in vivo* data (**MANUSCRIPT IV**) might implicate that Notch inhibition, like RA treatment (**MANUSCRIPT II**, Fig. 5), partly differentiate the bCSC, however, not enough to obtain detectable effects on the bCSC characteristics (**MANUSCRIPT III**, Fig. 5C-F) or decrease the tumor forming potential (**MANUSCRIPT IV,** Fig. 3B-D). However, if the treatment is sustained, as it was throughout re-plating in the soft-agar assay, the partly differentiated cells becomes proliferative exhausted and fail to form colonies (**MANUSCRIPT III**, Fig. 4B). However, if the treatment is not sustained, as was the case when the cells were intracranially injected in **MANUSCRIPT IV**, the cells might recapitulate their full potential by de-differentiation. The de-differentiation might not be back to the point of origin, at least not for all the cells, but instead to a bCSC sub-type with a different potential. This new potential might enable the bCSC to trans-differentiate into endothelial cells[16,316] promoting tumor angiogenesis leading to the abnormal and malignant looking vessels as observed in **MANUSCRIPT IV**, Fig. 4S-V. Thus, Notch inhibition, if not sustained, might select for a phenotype that more strongly induces angiogenesis. Indeed, Notch signaling has been implicated in tumor angiogenesis. E.g. Notch ligands have been demonstrated to be expressed by endothelial cells adjacent to Notch/Nestin positive GBM cells[317]. Furthermore, the Notch ligand Dll-4 has been shown to promote resistance to anti-angiogenic treatment, while inhibition of Notch signaling eliminated the resistance in an *in vivo* model[272] and reduced the number of endothelial cells in a 3D explant model[318]. Moreover, inhibition of Dll-4 has been shown to increase the number of abnormal, mal-functional vessel and decrease tumor size[319-321]. Finally, the significance of Dll-4-Notch signaling has been verified in patient material, as a sub-set of tumors showed increased activation of the pathway along with increased edema[273] indicative for leaky and malignant vessels[322]. It should however be emphasized that **MANUSCRIPT IV** is a manuscript in preparation and additional experiments needs to be conducted in order to solidify the manuscript conclusion: The micro array data, needs to be validated by real-time quantitative polymerase chain reaction. It would, moreover, be interesting to investigate if the abnormal vessels in the DAPT tumors are of murine or human origin, of which the latter would indicate trans-differentiation as discussed above. Finally, it would be interesting to test if sustained Notch (and/or Dll-4) inhibition throughout intracranial tumor formation, will hamper tumor growth and prolong the survival of the mice injected with high Notch expressing cells, as could be speculated based on our *in vitro* clonogenic assay (**MANUSCRIPT III**).

Overall the results obtained during this PhD project in some aspects support the literature with regard to the role of EGFR and Notch signaling in bCSC, while in other aspects we were not able to recapitulate the effect of treatment observed by others. Different setups and different inhibitors distinguish the studies, but also different GBM neurosphere cultures. As we observed differences in the gene expression profile as well as in the sensitivity to treatment between the cultures, this

project highlights the importance for stratified individual combination therapy that targets the different tumor cell populations from different angles, optimized for each sub-type.

8.3 Perspectives - combination of targeted therapy

8.3.1 EGFR-Notch interactions

The results presented in this PhD thesis indicate that EGFR/EGFRvIII and Notch signaling both play a role in bCSC maintenance and tumorigenicity and as such, to some degree play a similar role in the bCSC population. Indeed, as normal stem cells are pivotal for sustaining the organism, functional redundancy between several stem cell pathways, including the EGFR, and Notch pathways, ensure the possibility for maintenance of the stem cell population, and it is likely that this ability is present in the bCSC as well. This redundancy might be in terms of cross-talk between the pathways as reviewed in Katoh (2007)[323] and Doroquez and Rebay (2007)[324]. There are several examples on interactions between Notch and EGFR signaling during normal development and in different cancer types[324-329] and previous studies[262,330] together with preliminary results from our own laboratory (Olsen (2013)[311] and data not shown), indicate that this interaction exist in glioma and GBM as well. As described in section 2.5.3, activation of RAS alone or in combination with Notch induced expansion of Nestin positive cells and resulted in gliomagenesis in a mouse model, whereas Notch activation on its own failed to do so[230]. Even though the cell origin of bCSC has not yet been identified, increasing number of reports suggest that this role could be assigned to normal NSC. Thus, it could be speculated that increased EGFR signaling, either by amplification or mutation, drive oncogenic transformation in the NSC that exhibit endogenous Notch expression. Expansion of this, now tumor initiating bCSC population leads to increased Notch expression in the tumor. This hypothesis is supported by the sub-type studies discussed in section 2.1.1, as Notch pathway components were found up-regulated in the EGFR core[48]. Furthermore, Purow and colleagues have shown that Notch-1 knock down led to decreased activation of EGFR promoter activity and as such resulted in down regulation of the receptor, whereas activation of Notch resulted in EGFR up regulation[262]. Moreover, in the glioma cluster analysis displayed in Appendix 1b, AKT was down regulated upon DAPT treatment in all cultures, indicating a link between Notch signaling and a central signaling pathway downstream of EGFR. On the other hand, Notch independent Hes-1 up regulation has been demonstrated in gliomas and other tumors of the CNS and peripheral nervous system, possibly through a mechanism involving transforming growth factor (TGF)-α induced EGFR activation[329,330]. Thus, EGFR-Notch-1 cross-talk is a two way street, where both pathways regulate each other on different levels of the signaling cascade.

8.3.2 Implementation in the clinic

In the recent years, increased understanding of molecular abnormalities occurring in GBM has given rise to the development and use of targeted therapy in the search for an improved treatment, and optimally, a cure for this malignancy. Up until know, most targeted therapies have been aiming at a single molecule or pathway, deregulated in the cancer in question. One exception is the anti-angiogenic treatment, where different drugs target the same feature. The same could be the case when targeting the bCSC population. If different bCSC populations exists within the same tumor and/or in different tumors, all displaying treatment resistant and tumorigenic potential, and each

population has a unique expression profile as discussed in section 8.2.1, it could be tempting to speculate that each population rely on different signaling pathways in order to maintain their population, although redundant pathways also must be considered, as well as cross-talk between pathways as discussed above. As such, in order to target the different bCSC populations a combination of targeted therapies must be considered, also in order to inhibit redundant pathways that might result in treatment failure. If the goal is to differentiate the bCSC in order to sensitize them to conventional therapy one could either target different pathways in concert and/or combine this with a more general differentiation therapy such as RA. As outlined in section 8.2, RA treatment down regulates both EGFR/EGFRvIII (**MANUSCRIP II**) and Notch[271], again indicating these pathways to be important for maintaining the bCSC population and thus presenting a novel strategy for bCSC directed anti-GBM therapy.

As discussed in section 8.2.2, DAPT might not be the Notch inhibitor of choice as it, above off-target effects due to additional γ-secretase substrates, does not seem to fully inhibit Notch signaling. Alternatives to DAPT as a GSI inhibitor could be the GSI-18 or MRK-003 used in the study by Fan *et al*. of where MRK-003 seemed to be superior[26], although the unspecific nature of GSI still should be considered. Inhibiting Notch by different means than GSI could be the neural microRNA miR-326, that has been shown to be cytotoxic to bCSC *in vitro* and reduced tumor growth *in vivo*[331]. Several inhibitors (TKIs, mAbs, miRNAs, immunotoxins etc.) for EGFR have already emerged, some of which are in the clinic as outlined in section 2.4.3, and there are most likely more to come.

One of the major problems with the treatment today, is the resistance that inevitably leads to relapse and death. But by targeting the bCSC population it might be possible to prevent this. bCSC are believed to promote treatment resistance possibly as a result of endogenous expression of multidrug resistance pumps, DNA mismatch repair genes etc. as outlined in section 2.3.3. In line with this, it has been shown that GSI treatment, targeting the bCSC population, in combination with TMZ, targeting the tumor bulk, was superior to TMZ alone, when tested both *in vitro* on GBM neurosphere cultures and *in vivo* in a subcutaneous model[332]. Moreover, Notch inhibition has also been demonstrated to enhance the effect from radiation therapy[271,318]. If the EGFR and Notch pathways, to some extent, are redundant, it is possible that EGFR signaling likewise plays a role in treatment resistance. Indeed, a bCSC population expressing EGFR has been linked to resistance to chemo- and radiation therapy[46].

Taken together, targeting the bCSC population from different angles by using both specific signaling pathway targeted therapy, in concert with more a general differentiation therapy might increase the chance for targeting all the different bCSC populations and their redundant pathways in the tumor and thereby sensitize them to more conventional chemo- and radiation therapy. However, there is most likely a limit to the amount of anti-cancer drugs a GBM patient can tolerate, even though most of the treatment would be specifically targeted and thus less likely to result in severe side effects. Therefore, stratification of patients is necessary. As discussed in section 8.2.2, only patients that show increased expression and activation of a specific pathway should be stratified to therapy targeting the pathway in question, as we have shown was the case with Notch inhibition (**MANUSCRIPT III**). Sup-typing of GBM patients could be an effective tool for stratifying patients to the most optimal treatment. However, from a clinical point of view, this is not yet feasible, as no improved treatment for the individual sub-type can be offered at the moment. So for the time being,

when testing novel anti-EGFR and anti-Notch targets in the clinic, patients should be stratified by examining the expressed signature of the individual pathways.

9. Conclusion

The results included in this thesis support indications from previous published data, that a GBM model that supports the growth of NSC-like GBM cells, namely the bCSC, is an advanced model, when compared to traditional serum containing cultures of GBM. By transplanting patient GBM tumor tissue onto the flanks of nude mice and from there establish neurosphere cultures in serum-free media, we obtained an *in vivo*/*in vitro* model that maintained expression of amplified EGFR and the mutant variant EGFRvIII, as well as the expression of the Notch-1 receptor (section 8.1).
In addition, we show that EGFR and Notch signaling to some extent plays a role in the maintenance of bCSC and tumorigenic characteristics *in vitro*. We furthermore show that differentiating treatment of the neurosphere cultures down regulated EGFR/EGFRvIII expression, while others have showed the same being the case for Notch-1 expression as discussed in section 8.2. We did, however, not obtain an inhibitory effect on *in vivo* tumor growth from Notch inhibition, although we did observe histological changes in tumors formed from high Notch-1 expressing DAPT treated cultures.

Overall, the results obtained during this thesis project add to the existing literature on the subject and further implicate that EGFR and Notch signaling present promising targets for bCSC directed anti-GBM therapy. Whether the two signaling pathways in concert affects all bCSC in the tumor, or whether they each supports the growth and immature state of distinct bCSC subpopulations has not yet been clarified. However, it is likely that the two pathways exert redundancy through cross-talk by affecting the expression of the other receptor or by promoting downstream signaling independent of the default receptor as described in section 8.3.
Taken together, we suggest that an anti-GBM therapy that combines targeting of the bCSC populations by means of EGFR and Notch inhibition and differentiating therapy with conventional therapy that targets more differentiated tumor cells potentially could prevent the current inevitable relapse and thus improve the prognosis and survival of GBM patients with abnormal activation of the EGFR and Notch pathways.

10. References

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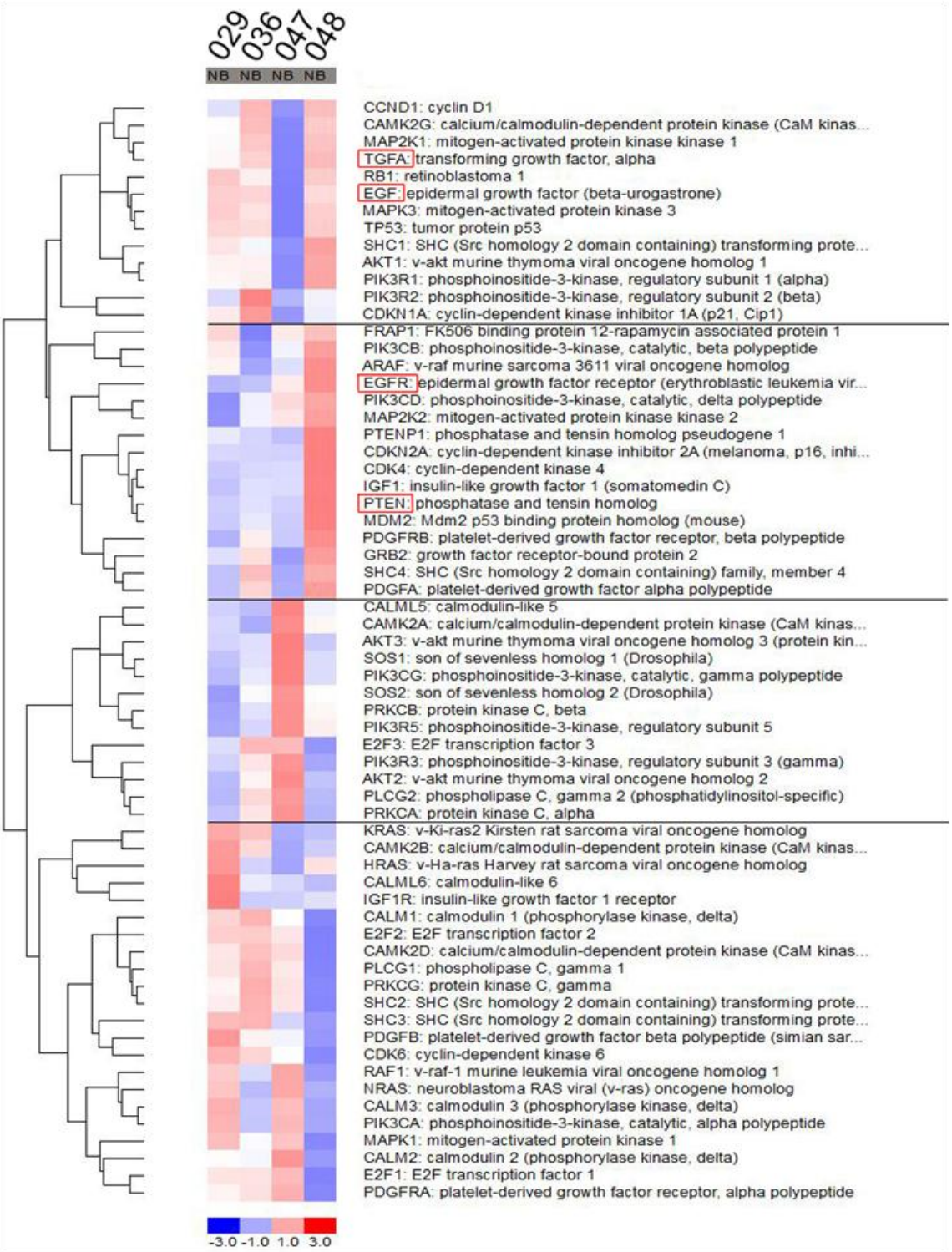
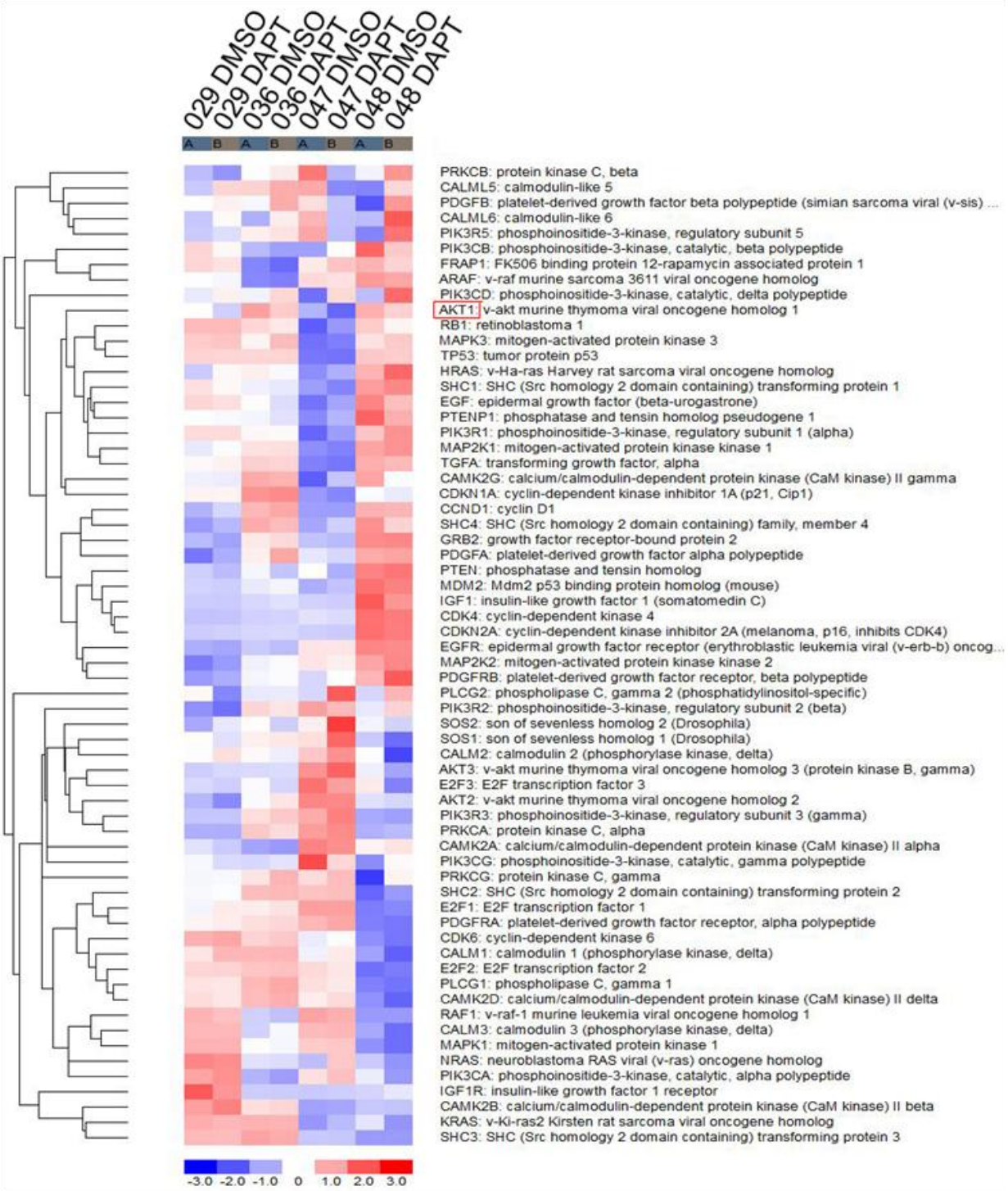
Appendix 1a: Glioma gene expression in NB cultures.



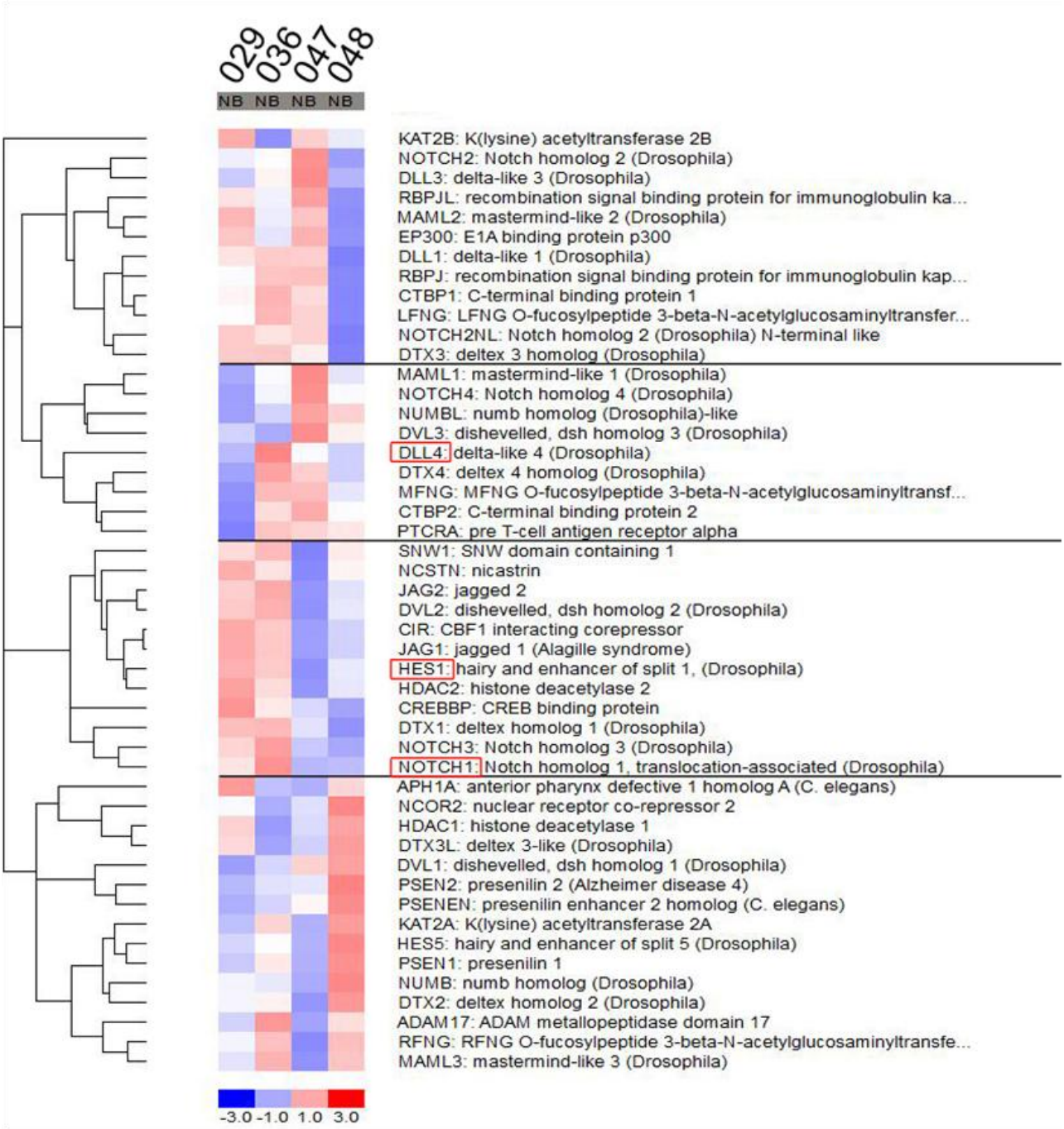
Gene expression analysis of KEGG_GLIOMA genes in the 029, 036, 047 and 048 neurosphere cultures. The expression level is relative to the mean expression of the respective gene in the four samples. 029: GBM_CPH029p7, 036: NGBM_CPH036p7, 047: NGBM_CPH047p3, 048: NGBM_CPH048p6. For material and methods, see **MANUSCRIPT IV**.

Appendix 1b: Glioma gene expression in DMSO and DAPT treated cultures.



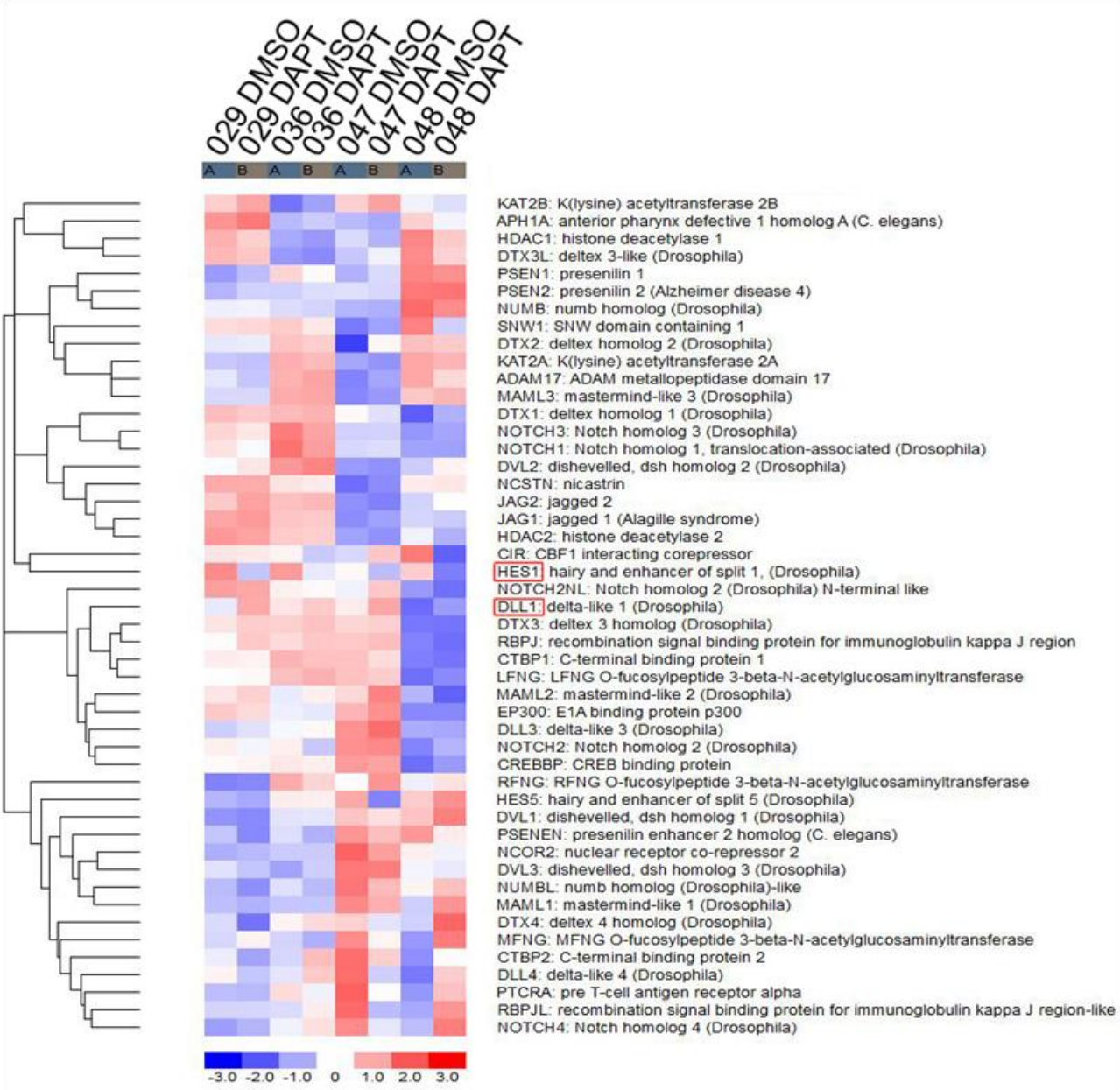
Gene expression analysis of KEGG_GLIOMA genes in the 029, 036, 047 and 048 neurosphere cultures treated with DAPT or DMSO for control. The expression level is relative to the mean expression of the respective gene in the four samples. 029: GBM_CPH029p7, 036: NGBM_CPH036p7, 047: NGBM_CPH047p3, 048: NGBM_CPH048p6. For material and methods, see **MANUSCRIPT IV**.

Appendix 2a: Notch pathway gene expression in NB-cultures.



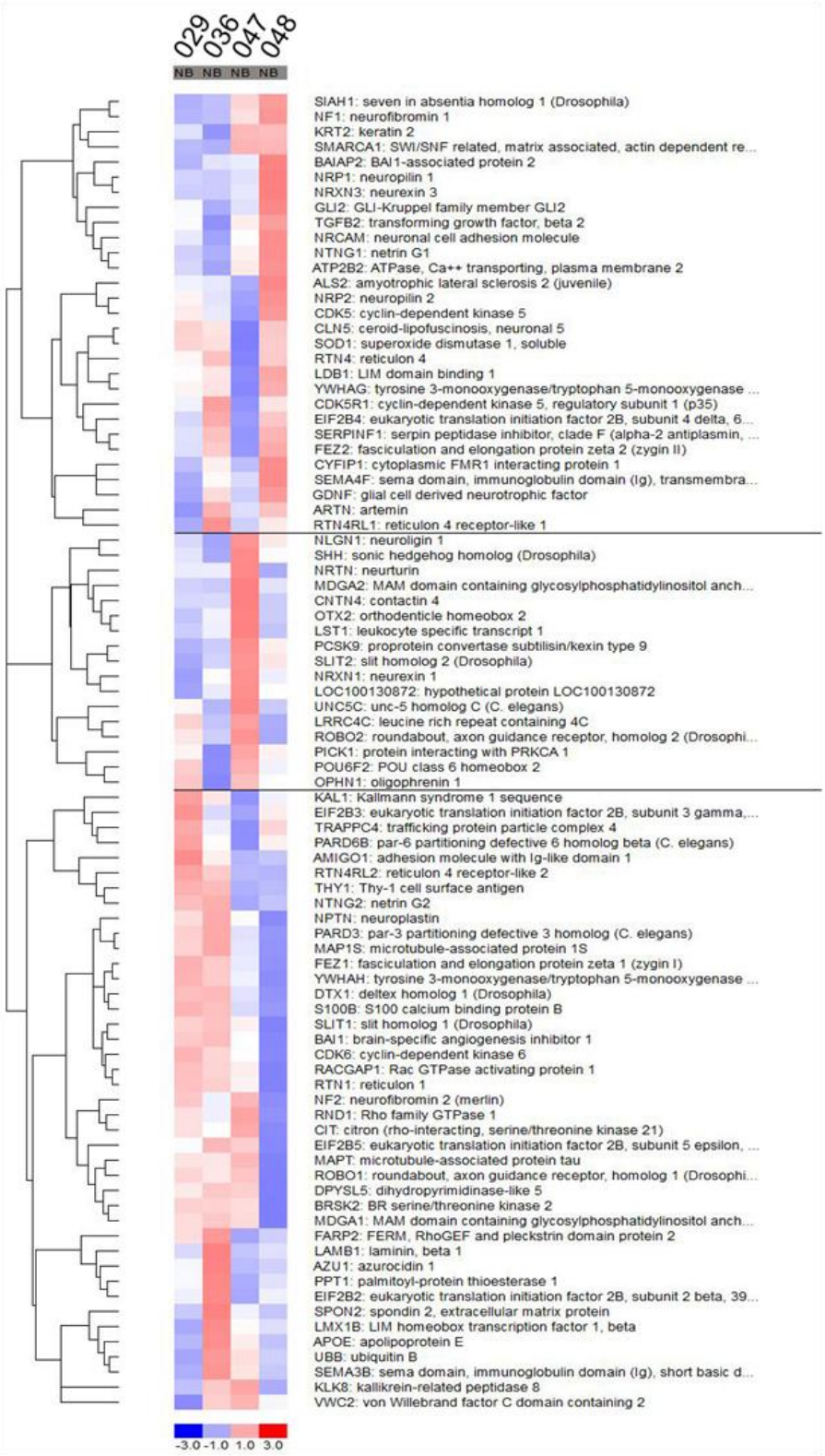
Gene expression analysis of KEGG_NOTCH_SIGNALING_PATHWAY genes in the 029, 036, 047 and 048 neurosphere cultures. The expression level is relative to the mean expression of the respective gene in the four samples. 029: GBM_CPH029p7, 036: NGBM_CPH036p7, 047: NGBM_CPH047p3, 048: NGBM_CPH048p6. For material and methods, see **MANUSCRIPT IV**.

Appendix 2b: Notch pathway gene expression in DMSO and DAPT treated cultures.



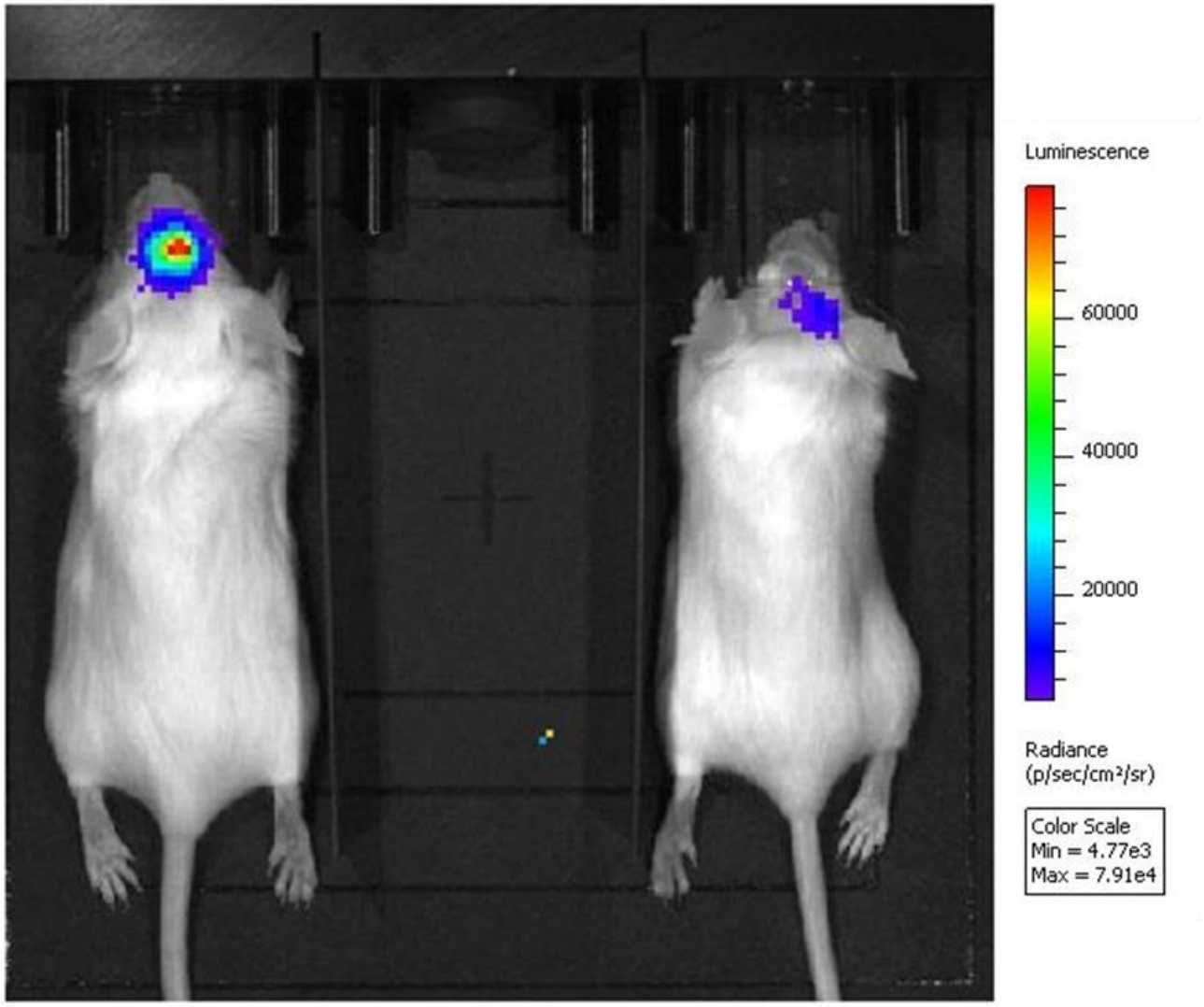
Gene expression analysis of KEGG_NOTCH_SIGNALING_PATHWAY genes in the 029, 036, 047 and 048 neurosphere cultures treated with DAPT or DMSO for control. The expression level is relative to the mean expression of the respective gene in the four samples. 029: GBM_CPH029p7, 036: NGBM_CPH036p7, 047: NGBM_CPH047p3, 048: NGBM_CPH048p6. For material and methods, see **MANUSCRIPT IV**.

Appendix 3: Neurogenesis gene expression in NB- cultures.



Gene expression analysis of NERVOUS_SYSTEM_DEVELOPMENT genes in the 029, 036, 047 and 048 neurosphere cultures. The expression level is relative to the mean expression of the respective gene in the four samples. 029: GBM_CPH029p7, 036: NGBM_CPH036p7, 047: NGBM_CPH047p3, 048: NGBM_CPH048p6. For material and methods, see **MANUSCRIPT IV**.

Appendix 4: Bio-luminescence detection of an intracranial xenograft tumor.



Bio-luminescence detection of an intracranial xenograft tumor in two SCID mice. The image is generated six days after intracranial injection of luciferase transfected 048p6 cells, by administrating the substrate luciferin *i.p.* approximately 20 minutes prior to acquisition under gas anesthesia. Image kindly lend from Mette Kjølhede Nedergaard.

Declaration of co-authorship

This declaration concerns the article: *Maintenance of EGFR and EGFRvIII expressions in an in vivo and in vitro model of human glioblstoma multiforme*.

A part of the thesis: *A functional study of EGFR and Notch signaling in brain cancer stem-like cells from glioblastoma multiforme*.

Submitted for the defense/obtainment of the PhD degree at the University of Copenhagen.

Name of the PhD student: Karina Kristoffersen

The proportion of the PhD students contribution to the article in question is evaluated from the following scale:

A. Has contributed to the co-operation (0-33%)
B. Has contributed considerably to the co-operation (34-66%)
C. Has predominantly executed the work independently (67-100%)

Declaration regarding specific elements (1.-4.)	Extent (A,B,C)
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable via experiments.	A
2. Planning of the experiments and methodology design, including selection of methods and method development.	A
3. Involvement in the experimental work.	B
4. Presentation, interpretation and discussion in a journal article format of the obtained data.	A

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2. Planning of the experiments and methodology design, including selection of methods and method development.	C
3. Involvement in the experimental work.	C
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2. Planning of the experiments and methodology design, including selection of methods and method development.	C
3. Involvement in the experimental work.	C
4. Presentation, interpretation and discussion in a journal article format of the obtained data.	C

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PhD Thesis

Karina Kristoffersen, MSc

A Functional study of EGFR and Notch signaling in brain cancer stem-like cells from glioblastoma multiforme