Angiogenesis Inhibition in Glioblastoma

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Angiogenesis Inhibition in Glioblastoma

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
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Chapter 1

Introduction and general outline

Angiogenesis inhibition in glioblastoma:

The discovery of angiogenesis inhibition

Blood vessels constitute the first organ in the embryo and form the largest network in our body. Small blood vessels consist only of endothelial cells, whereas larger vessels are surrounded by pericytes and smooth muscle cells. Blood vessels can grow in several ways: Vasculogenesis refers to the formation of blood vessels by endothelial progenitors and Angiogenesis refers to the physiological process through which new blood vessels form from sprouting from pre-existing vessels and subsequent stabilization of these sprouts. When dysregulated, the formation of new blood vessels contributes to numerous malignant, ischemic, inflammatory and infectious disorders¹ and inducing angiogenesis is considered one of the six hallmarks of cancer². Since its first description as an therapeutic target in oncology by Judah Folkman in 1971 angiogenesis has been extensively studied in numerous types of cancer³. Currently the “angiogenic switch”, when the balance between pro- and anti-angiogenic factors tilts towards a pro-angiogenic outcome, is considered a rate-limiting secondary event in multistage carcinogenesis⁴. The accepted consensus is, that this pro-angiogenic outcome leads to a transition from dormant avascularized hyperplasia to outgrowing vascularized tumor. In the first classical description by Dr Folkman on targeting this angiogenesis, anti-angiogenic agents would have a direct effect on endothelial cell apoptosis, which lead to cessation of new blood vessel growth³. This process ideally would lead to less oxygen and nutrients to the tumor and ideally to tumor “starvation”. But as the understanding of angiogenesis advanced it became clear that there are more pathophysiological mechanisms that contribute to this phenomenon.

Targeting angiogenesis: The discovery of VEGF

After the discovery of anti-angiogenesis as a new anticancer strategy, several molecules that can induce blood vessel growth in various bioassays were identified. Those included fibroblast growth factor 1 (FGF1; also known as aFGF), bFGF, angiogenin and transforming growth factor- α (TGF α), but their role in the regulation of angiogenesis remained unclear⁵. In 1989, the isolation and cloning of vascular endothelial growth factor A (VEGFA, previously known as vascular permeability factor (VPF))⁶⁷ was a major step forward in understanding angiogenic mechanisms. Several studies showed that VEGFA possessed strong angiogenic properties that made it an attractive target for anti- angiogenesis⁸. In 1993, Kim and colleagues identified monoclonal antibodies that can target and neutralize VEGFA and inhibit tumor growth in preclinical studies⁹. The authors were able to demonstrate that when they injected human rhabdomyosarcoma, glioblastoma or leiomyosarcoma cell lines into nude mice this monoclonal antibody inhibited the growth of the tumors, but had no effect on the growth rate of the tumor cells *in vitro*. This led to the production of the recombinant humanized VEGFA-specific monoclonal antibody

bevacizumab (Avastin; Genentech/Roche), which was approved in 2004 by the US Food and Drug Administration (FDA) for the first-line treatment of metastatic colorectal cancer¹⁰. While it has been utilized in clinical practice for multiple indications since then, it is becoming clear that it has variable effectiveness in different types of cancer one of which being glioblastoma¹¹.

Angiogenesis in glioblastoma

The prognosis of the malignant brain tumor glioblastoma multiforme remains poor. After surgical resection, the standard of care for patients with newly diagnosed glioblastoma and a good Karnofsky performance score is concurrent radiotherapy and temozolomide, followed by adjuvant temozolomide¹². Despite this intensive treatment, no further improvements in outcomes have been documented since the introduction of radiotherapy–temozolomide therapy in 2005 and the median survival remains around 15 months. Due to its highly vascularized nature, the malignant brain tumor glioblastoma was hoped to be an ideal target for anti-angiogenic therapies like bevacizumab. In the first trials with bevacizumab in glioblastoma, enthusiasm was generated due to the radiographic response to this treatment, meaning the reduction in contrast enhancement on MRI, next to the reduction in tumor associated edema but unfortunately it did not correlate with the tumor response^{13,14}. Two phase III trials in 2014 showed that adding bevacizumab to standard chemoradiotherapy only improved progression free survival without any effect on overall survival^{15,16}. Additionally, a meta-analysis of randomized controlled trials in the recurrent setting of bevacizumab combined with chemotherapy showed an effect only on progression-free survival¹⁷. Nonetheless bevacizumab continues to be used in some settings, especially in recurrent tumors that have failed standard therapies and/or that do not qualify for targeted therapies. This lack of benefit in randomized trials, while clear benefit in the perception of clinicians due to the clear radiographic response and the reduction of tumor associated edema that improved patients clinical status, has resulted in increased effort in continuing the investigation of the mechanisms of antiangiogenic therapy with the goal to identify individual patients that would benefit most from this treatment.

Understanding the mechanism of failure

Because of the failure to find survival benefit after treatment with bevacizumab in phase III trials, increased effort into the understanding the mechanisms underlying this resistance has been undertaken. As the understanding of angiogenesis advanced it became clear that there are more pathophysiologic mechanisms that contribute to this phenomenon, such as vascular co-option, vasculogenesis, and vascular mimicry with all distinct mechanisms and cell signaling pathways that all play important roles in resistance to anti-angiogenic treatment. Although a minority of tumors seems to be intrinsically non-responsive to anti-angiogenic therapy, most tumors

employ evasive resistance mechanisms after an initial response phase. These evasive mechanisms that glioblastoma use include activation or upregulation of alternative pro-angiogenic signaling pathways within the tumor, recruitment of bone marrow derived pro-angiogenic cells, increased pericyte coverage of the tumor vasculature and activation and enhancement of invasion¹⁸. Although induction of angiogenesis is accepted as an hallmark of cancer, it is important to realize that the primary effect of anti-VEGF therapy is not cytotoxic and there is no evidence that bevacizumab directly induces glioma cell death. The main effect is rather pruning of abnormal tumor vasculature which leads to vascular normalization¹⁹. The resulting hypoxia that is exacerbated by these therapy most likely initiates a cascade of events that ultimately does not stop tumor growth with the current anti-angiogenic agents. Additionally, anti-VEGF therapy only blocks a single growth factor in a complex process of angiogenesis and thus only one aspect of the highly heterogeneous glioblastoma micro-environment in the setting of multiple events that contribute to angiogenesis and tumor growth. As mentioned earlier there are multiple stages within the tumor microenvironment that lead to blood vessel growth that are dependent on several other growth factors and are therefore most likely not affected by just one treatment²⁰. This has led to an increased focus on the tumor microenvironment and the way treatment resistance mechanisms are being employed.

Central hypothesis

Despite these encountered difficulties and complexity of the tumor micro-environment, I am convinced that anti-angiogenic therapy for glioblastoma can be very effective when the right angiogenesis inhibitor and combination treatment is found. This has led to the central hypothesis: **Angiogenesis inhibition is an effective treatment for patients with glioblastoma**. The goal is to be able to identify patients that will benefit the most from this treatment and find research models that can answer the question which evasive mechanisms glioblastoma uses in the tumor micro-environment to become resilient to anti-angiogenic therapy and find treatment modalities to overcome this resistance.

To test this central hypothesis, I have generated five sub-hypotheses, which are described below and form the topic of investigation of the five chapters.

In chapter 1, I tested the hypothesis that radiation increases VEGF secretion in glioblastoma cell lines. In this *in vitro* study, I will measure the VEGF secretion by ELISA in glioblastoma cell lines. The combination of an angiogenesis inhibitor and radiation is possibly a more effective treatment. In chapter 2, I tested the hypothesis that

the molecular profile by TCGA classification can predict response to bevacizumab in glioblastoma patients. Using a retrospective approach, I will analyze the response to bevacizumab in

patients with recurrent glioblastoma. The goal is to identify patients that are most likely to respond to this treatment. In [Chapter 3](#), we tested the hypothesis that brain tumor stem cells in glioblastoma can form endothelial cells. In this *in vitro* and *in vivo* study, we will analyze an alternative way in which glioblastoma can form blood vessels. This may explain why anti-angiogenic therapy has variable effect. In [Chapter 4](#), I tested the hypothesis that brain tumor stem cells in glioblastoma can be studied in an organotypic “explant” culture system. In this *in vitro* study, I aim to find a clinically relevant model to study brain tumor stem cells in the tumor micro-environment. In [Chapter 5](#), I tested the hypothesis that Notch inhibition inhibits brain tumor stem cells and angiogenesis in an organotypic “explant” model of glioblastoma. In this *in vitro* study, our aim is to inhibit brain tumor stem cells in a clinical relevant model by using a known inhibitor of neural stem cells. This can potentially be used to overcome the resistance to the more commonly used angiogenic inhibitors.

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2

Chapter 2

Radiation-enhanced vascular endothelial growth factor (VEGF) secretion in glioblastoma multiforme cell lines – a clue to radioresistance?

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Key words: brain tumor, glioblastoma multiforme, ionizing radiation, radioresistance, vascular endothelial growth factor, VEGF

Objective: Postoperative radiotherapy is standard treatment for patients with a glioblastoma multiforme (GBM). However, a GBM is radioresistant and almost always recurs, even after a high dose of radiation. A GBM is characterized by its extensive neo-angiogenesis, which can be attributed to the high levels of vascular endothelial growth factor (VEGF). The scope of this study is to investigate the VEGF secretion by GBM cells with different radiosensitivity after irradiation.

Methods: Three human GBM cell lines (U251, U251-NG2 and U87) were irradiated with single doses of 0, 5, 10 and 20 Gy of γ -rays from a ^{137}Cs source. VEGF levels in medium were measured by ELISA at 24, 48 and 72 h after radiation. Cell survival was measured by the XTT assay 7 days after irradiation.

Results: Following single dose radiation, the VEGF levels showed a dose dependent increase in U251, U251-NG2 and U87 glioma cells. Both base-line and radiation-enhanced VEGF levels were about 10-fold higher in U87 compared to U251 and U251-NG2 cells. In addition, in the XTT assay, the U87 was more radioresistant than both U251 and U251-NG2 cell lines (dose modifying factor (DMF) 1.6 and 1.7 resp.).

Conclusion: Irradiation enhanced VEGF secretion in all three tested glioma cell lines (up to eight times basal levels). It is tempting to associate the radiation-enhanced VEGF secretion with an increased angiogenic potential of the tumor, which may be a factor in radioresistance.

Introduction

The mean survival time of patients suffering from the very malignant GBM is 1 year. In addition to operation the standard treatment consists of radiotherapy. Unfortunately local recurrence occurs in more than 90% of patients.

In the progression from low-grade tumors to more anaplastic tumors, gliomas show an increasing vessel density. The final transition to glioblastoma is marked by a characteristic endothelial proliferation and further increased vessel density, which often takes the form of glomeruloid vascular structures. This so called 'angiogenic switch' is associated with an up-regulation of VEGF and induction of its tyrosine kinase receptors flt-1 and flk-1/KDR in endothelial cells [1]. Indeed significantly elevated levels of VEGF were found in the tissue and cyst fluid of glioblastomas and VEGF concentration clearly correlated with the vascularity of these glioblastomas [2]. Even in tumor cells infiltrating the surrounding brain tissue, a high expression of VEGF was found [3]. Extensive studies on VEGF and its receptors over the past 10 years have revealed that this system is a major regulator for angiogenesis [4]. VEGF has also been reported to have direct stimulatory growth and survival effects on neurons and glial cells [5]. Furthermore, VEGF has been shown to act as a survival factor for several tumor cells as well as normal cells by protecting against apoptosis-inducing treatments, such as chemotherapy and irradiation [6]. With respect to endothelial cells, VEGF reduced the damaging effects of irradiation which may limit the vascular damage induced by radiotherapy [7–9]. Therapeutic options for glioblastoma multiforme treatment might be present with the observation that blocking VEGF by a monoclonal antibody in a heterotopic mice tumor model led to a reduced tumor growth [10]. Inhibition of the VEGF receptor, Flk-1, also induced complete inhibition of established glioblastoma xenografts [11].

The observation that serum VEGF is increased after radiotherapy in brain tumor patients [12], suggests that the VEGF pathway is activated by radiation. In fact, Gorski [7] showed that in a number of human tumor cell lines a single dose of 10 Gy irradiation induced enhanced VEGF secretion after 24 h. The enhanced VEGF secretion by surviving tumor cells may increase survival by decreasing apoptosis, stimulating proliferation and increasing angiogenic potential [6–9]. These factors may all contribute to the high vascularity as well as the high radioresistance of glioblastoma multiforme.

To establish whether enhanced VEGF secretion after irradiation is a general response of GBM cells, we studied the VEGF secretion in response to irradiation of two commonly used GBM cell lines, one with a high basal level of VEGF production (U87) [7] and one with a low basal VEGF level (U251) [13]. Additionally, the U251 GBM cell line transfected with the proteoglycan NG2 (U251-NG2) was studied, since this cell line was shown to form highly vascular tumors, although the tumor is a low VEGF secretor [13].

Methods

Cell culture and selection of NG2-expressing cells

U251 and U251-NG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Life Technologies, Paisley, Scotland), supplemented with 10% heat-inactivated newborn calf serum, four times the prescribed concentration of essential amino acids, 2% glutamine, penicillin (100 IU/ml), and streptomycin (100 µg/ml). This was supplemented with geneticin disulfate (800 µg/ml) (Sigma, Dorset, UK) to select for neomycin-resistant clones.

U87 cells were maintained in Dulbecco's modified Eagles's medium (DMEM) (GIBCO Life Technologies, Paisley, Scotland), supplemented with 10% fetal calf serum, 2% glutamine, penicillin (100 IU/ml), and streptomycin (100 µg/ml).

Irradiation

Because only one of the three cell lines formed colonies, the clonogenic assay, which is the standard for determining radiosensitivity, could not be used. In order to compare the radioresistance of all three cell lines we used a proliferation-based assay (XTT assay), which is highly comparable to the clonogenic assay when the cells are allowed to undergo six cell division [14]. The three cell lines were radiated with γ -rays from a ^{137}Cs source at a dose rate of about 0.8 Gy/min. After irradiation with 0, 2, 4, 6, or 8 Gy, the cells were plated in well plates (100 µL cell suspension, 500 and 1000 cells per well). After 7 days, the number of living cells was determined by a proliferation XTT assay (Roche Molecular Biochemicals, Mannheim, Germany); linearity range absorbance [A492–A690 nm]: 0.5–2.5; minimal detection limit approximately 5000 cells per well). In short, 50 µl of the substrate (sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-meth-oxy-6-nitro) benzene sulfonic acid hydrate) was added and incubated for 4 h at 37 °C. The substrate is converted into an orange formazan dye only in metabolically active cells. The formazan dye is soluble in aqueous solutions and can be measured using a spectrophotometer. Growth inhibition curves were prepared by the relative growth based on the optical densities of treated and control wells and IC_{50} values were determined.

Measurement of VEGF levels

The cells were plated in six-well plates at 25% confluence and allowed to attach overnight. They were irradiated with 0, 5, 10 and 20 Gy using a ^{137}Cs source. Conditioned medium was collected 24, 48 or 72 h after irradiation and the number of cells in each well was determined using a hemocytometer. Debris was pelleted for 5 min at 2000 rpm and supernatant was collected and stored at -20°C .

A dilution series sequence of the samples with the highest and lowest expected values of VEGF was first performed to determine the appropriate dilutions. VEGF levels were measured by ELISA using an ELISA- kit (Biosource International, Camarillo, California, USA) according to manufacturers guidelines and normalized to cell number in each well. Levels of VEGF were

expressed as pg/ml/ 10^6 cells. From the cumulative measured values the VEGF-secretions by day were calculated. All standards, controls and samples were performed in duplicate. All experiments were done in triplicate.

Data analysis

Differences were analyzed with the Student *t* test. *P* values below 0.05 were considered to be significant.

Results

Effect of irradiation on VEGF production in GBM cell lines

Following single dose radiation, the VEGF levels showed a dose dependent increase in both U251, U251-NG2 and U87 (Figure 1) ($P < 0.05$). Maximal increase was seen 72 h post irradiation in all three GBM cell lines. The levels of VEGF showed an radiation-induced increase after 24 h. A statistically significant increase ($P < 0.05$) in VEGF secretion was observed for each dose and each time point except for U251 and U251-NG2 treated with 5 Gy after 72 h (respective P 0.7998, P 0.6312), U87 treated with 5 Gy after 24 h (P 0.8312) and U251 after 10 Gy and 72 h (P 0.0755). The highest levels of VEGF were found after 72 h in the medium of cells irradiated with 20 Gy (U251: 16784, U251-NG2: 20992, U87: 112227 pg/ml/ 10^6 cells).

Of the three cell lines tested here, U87 produced the most VEGF both with and without irradiation (U87: 13.936 pg/ml/ 10^6 cells, U251; U251-NG2: 1422 and 1240 pg/ml/ 10^6 cells

Figure 1. VEGF protein levels (pg/ml/ 10^6 cells) in U251 (white bar), U251-NG2 (grey bar) and U87 (black bar) conditioned medium after irradiation. Mean with standard errors of three separate experiments performed in duplo are shown. U87, U251 and U251-NG2 cells were plated in six-well plates at 25% confluence, allowed to attach overnight, and then irradiated with 0, 5, 10 or 20 Gy. Conditioned medium was collected every 24 h, and VEGF levels were normalized to cell number.

Figure 2. VEGF protein levels (pg/ml/ 10^6 cells) in U251 (a), U251- NG2 (b) and U87 (c) conditioned medium *per day* after irradiation. Mean with standard errors of three separate experiments performed in duplo are shown. VEGF-secretion by day was calculated from the cumulative data.

Figure 3. XTT assay of U251 (square), U87 (diamond) and U251- NG2 (triangle) ($n = 4$). The three cell lines were irradiated with 0, 2, 4, 6, and 8 Gy. After 7 days, the number of living cells was determined by a proliferation XTT assay. Growth inhibition curves were prepared by the relative growth based on the optical densities of treated and control wells from which IC_{50} values were derived.

after 24 Gy and no irradiation). U251 and U251-NG2 cells produced similar amounts of VEGF with and without irradiation. A dose dependent, radiation-induced, increase of VEGF was shown in these cell lines as well. This increase was already apparent after 24 h and maximum after 72 h.

The total VEGF-secretion per day showed a higher variability than the cumulatively measured values (Figure 2 A–C). The secretion of VEGF per day after a single dose of radiation was higher

than without irradiation for most values of the three cell lines: U251, U251-NG2 and U87. The increase of VEGF secretion per day was up to eight times over control. Generally, the VEGF-secretion per day increased in a dose-dependent manner, with maximum increase after irradiation with a single dose of 20 Gy.

Sensitivity to irradiation of the GBM cell lines U87, U251 and U251-NG2

To determine the sensitivity of the three cell lines to irradiation a XTT assay was performed on all three cell lines. Growth inhibition curves were prepared by using the relative growth (see materials and methods) (Figure 3). U87 cells were more resistant to irradiation than either U251 cells or U251-NG2. U251 and U251-NG2 cells had similar sensitivities to irradiation. At the IC_{50} U87 cells were more resistant to irradiation than U251 and U251-NG2. The dose modifying factor (DMF) for U251 is 1.6 compared to U87 and the DMF for U251-NG2 is 1.7 compared to U87.

Discussion

This study shows that radiation induces a dose-dependent increase in VEGF-secretion in three glioblastoma multiforme cell lines: U87, U251 and U251-NG2. The effect of radiation was most apparent after a single dose of 20 Gy. The increase in VEGF secretion per day was up to eight times for the three tested cell lines. It is unlikely that the measured increase in VEGF concentration in the conditioned medium does not reflect enhanced secretion. We did not find a decrease in cell number after irradiation, even after irradiation with 20 Gy (data not shown). Other studies also failed to demonstrate apoptosis or autophagy in a number of glioblastoma cell lines after irradiation [15]. Moreover, VEGF has been shown to reduce apoptosis after irradiation in human leukemia cells and in human and murine mammary adenocarcinoma cells [16–18]. Our results are similar to the results obtained by Gorski and coworkers on Lewis lung carcinoma (LLC) cells [7]. The LLC cells also showed a dose-dependent, time dependent increase in VEGF secretion after irradiation. The radiation-enhanced VEGF secretion has so far been shown in GBM cell lines (U87, T98, T9 RT2), a lung carcinoma cell line (LCC), an oesophageal adenocarcinoma (Seg-1), a squamous cell carcinoma (SQ20B), a melanoma cell line (U1) and primary astrocytes [7,19]. Irradiation has been shown to stimulate the VEGF promoter via multiple mitogen activated protein kinase (MAPK) dependent pathways in primary astrocytes and GBM cell lines [19]. Hypoxia seems not to be involved in this mechanism, since the hypoxia-inducible factor HIF1 α was not expressed after irradiation [19]. However, progression elevated gene 3 (PEG 3) may be involved in the observed enhancement of VEGF secretion, because irradiation activated the PEG-3 promoter via MAPK signalling in primary astrocytes and GBM cell lines [19]. In the glioblastoma cell lines T9 and RT2 the PEG-3 and VEGF expression correlated directly to their oncogenic potential [19].

In the present study an XTT assay was performed on U251, U251-NG2 and U87 cells to assess the relative sensitivity to irradiation, in comparison with each other. U251 and U251-NG2 showed similar sensitivity. At the IC_{50} -level U87 cells were more resistant to irradiation than U251 and U251-NG2 (DMF: 1.6 and 1.7 resp.). These results are in concordance with the radiobiological parameters of U87 and U251 found by Haas- Kogan et al. in clonogenic assays [20].

Since U87 was most resistant to irradiation and had the highest basal VEGF secretion, this suggests that enhanced VEGF secretion is involved in radioresistance. Steiner et al. showed that the upregulation of VEGF leads to a downregulation of glioblastoma cell proliferation, which might explain the reduced radiosensitivity [21]. The role of VEGF in radioresistance is supported by preclinical studies, in which GBM cell line cells were placed in a murine tumor model system and treated with radiotherapy in combination with anti-VEGF therapy [7]. A greater than additive effect was seen [7–9,22], which was independent of the degree of hypoxia [23]. The importance of irradiation induced *enhanced* VEGF- secretion in radioresistance was not studied so far. In further studies we plan to investigate the importance of the radiation-induced enhancement of VEGF secretion (e.g. U251 and U251-NG2) in an orthotopic mouse tumor model.

In conclusion, irradiation induces significant enhancement of VEGF-secretion which appears to be a general response in GBM cell lines. Therefore we speculate that the radiation-enhanced VEGF-secretion decreases apoptosis and increases angiogenesis, both leading to GBM radioresistance.

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3

Chapter 3

EGFR amplification and classical subtype are associated with a poor response to bevacizumab in recurrent glioblastoma

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Purpose The highly vascular malignant brain tumor glioblastoma (GBM) appears to be an ideal target for anti-angiogenic therapy; however, clinical trials to date suggest the VEGF antibody bevacizumab affects only progression-free survival. Here we analyze a group of patients with GBM who received bevacizumab treatment at recurrence and are stratified according to tumor molecular and genomic profile (TCGA classification), with the goal of identifying molecular predictors of the response to bevacizumab.

Methods We performed a retrospective review of patients with a diagnosis of glioblastoma who were treated with bevacizumab in the recurrent setting at our hospital, from 2006 to 2014. Treatment was discontinued by the treating neuro-oncologists, based on clinical and radiographic criteria. Pre- and post-treatment imaging and genomic subtype were available on 80 patients. We analyzed time on bevacizumab and time to progression. EGFR gene amplification was determined by FISH.

Results Patients with classical tumors had a significantly shorter time on bevacizumab than mesenchymal, and proneural patients (2.7 vs. 5.1 vs. 6.4 and 6.0 months respectively, $p = 0.011$). Classical subtype and EGFR gene amplification were significantly associated with a shorter time to progression both in univariate ($p < 0.001$ and $p = 0.007$, respectively) and multivariate analysis (both $p = 0.010$).

Conclusion EGFR gene amplification and classical subtype by TCGA analysis are associated with significantly shorter time to progression for patients with recurrent GBM when treated with bevacizumab. These findings can have a significant impact on decision-making and should be further validated prospectively.

Keywords Bevacizumab · Classical · EGFR · Glioblastoma · Mesenchymal · Proneural

Introduction

Since its description in 1993 the monoclonal antibody against VEGF (bevacizumab) has been extensively studied [1, 2], and is in use for several cancer types. Due to its highly vascularized nature, glioblastoma was once considered an ideal target for anti-angiogenic therapies like bevacizumab (Avastin) [3]. However, data emanating from clinical trials as well as off trial experience have shown a modest and unsustained impact on disease progression. Two recent phase III trials showed that adding bevacizumab to standard chemo-radiotherapy in the upfront setting only improved progression-free survival with no overall survival benefit [4, 5]. A recent meta-analysis of randomized controlled trials of bevacizumab combined with chemotherapy in the recurrent setting also showed, an effect only on progression-free survival [6]. Nonetheless bevacizumab continues to be used in some settings, especially in recurrent tumors that have failed standard therapies and/or that do not qualify for targeted therapies. In view of increasing drug costs, the complications of the treatment and the lack of demonstrated survival benefit, identification of potential predictors of bevacizumab response, beyond common factors such as KPS could help guide treatment decisions [7].

Additional experience with bevacizumab has allowed for clarification of complications of treatment in GBM that include thrombocytopenia, cerebral hemorrhages, arterial thrombo-embolic events, proteinuria, hypertension, visceral perforation, and inhibition of wound healing [8–12]. Furthermore, as the experience with this therapy grew, a more invasive pattern of progression of GBM has been observed to develop while under anti-angiogenic therapy [13, 14]. A potential mechanism involving the inhibition of the HIF1 α mediated hypoxic response in the tumor micro-environment has been proposed to underlie the more invasive or multifocal phenotype [15]. In light of these potential downsides, identifying which patients will benefit the most from bevacizumab treatment is crucial. We hypothesized that stratification by molecular profile, data that is increasingly available at many institutions, might provide a basis for prediction of treatment response, and eventually a rational parameter to be used for selecting patients to be treated with bevacizumab.

Recent large-scale genomic analyses have revealed patterns of molecular changes within tumor subclasses that harbor distinct underlying biology and clinical prognosis [16, 17]. Over the last decade or so, the TCGA (The Cancer Genome Atlas) classification was adopted by many centers, including ours. According to this schema, glioblastomas can be classified by transcriptomal features into classical, mesenchymal, neural or proneural subtypes that exhibit different gene mutation profiles, biological properties and treatment responses [17]. In addition, several well-known molecular markers such as IDH-1 mutation, EGFR amplification or mutation, and MGMT promoter methylation are often reported in GBM [18]. Molecular subtyping is increasingly incorporated into more traditional schemes, such as the latest WHO classification update [19]. The clinical challenge for the future will be to match treatment to the best molecular fit.

An earlier post-hoc analysis of the BELOB trial determined that tumors classified as “classical subtype” per TCGA criteria at first diagnosis, when treated with bevacizumab and CCNU at

recurrence), led to an improvement in progression free survival [20]. Notably this cohort had almost exclusively classical tumors. In this report we sought to determine if molecular subclasses of glioblastoma (determined at the time of recurrence) respond in a distinct manner to bevacizumab treatment when given at recurrence.

Methods

Patient selection

Patients with pathologically confirmed glioblastoma treated with bevacizumab (Genentech South San Francisco, California, United States) in the recurrent setting and for whom genomic subtype analysis according to TCGA criteria (classical, proneural, mesenchymal, and neural) was available, were retrospectively identified. The classification was performed soon after tissue acquisition, independently of treatment decisions, and confirmed by CB and JH. Patients meeting these criteria treated at our hospital between 2006 and 2014 were retrieved by a search of a prospectively collected brain tumor registry database. The research protocol was approved by the IRB. KPS, gender, age, date of death and other treatment modalities were collected.

Molecular data

All molecular data was determined at recurrence. EGFR amplification was determined by FISH analysis, EGFR VIII by immunostaining, and MGMT promoter methylation status by real-time methylation specific PCR at our institution. IDH mutation analysis has been available on more recent cases but was not available on enough patients to do a meaningful analysis.

Nanostring analysis

Analysis of the Cancer Genome Atlas (TCGA) glioblastoma transcriptional subclasses was determined by using a semiquantitative gene expression profiling assay (Nanostring nCounter, Seattle, Washington, USA) based on 81 genes selected from the initial TCGA publication as described earlier [17, 21, 22]. Briefly, RNA from 192 GBM samples from TCGA was analyzed with codeset of 146 probes, including for 81 genes previously selected to distinguish TCGA expression subclasses. Raw codeset counts were normalized by a panel of GBM-invariant genes [22] and were used to generate centroids for four transcriptomal classes (proneural, classical, mesenchymal and neural) according to published classification by TCGA [17]. RNA was extracted from tumor tissue and assayed by the same Nanostring protocol and codeset. Patient sample RNA transcriptomal class assignments were assigned by the nearest TCGA class centroid by correlation.

Outcomes

Our main outcome measurement was time to progression on bevacizumab, defined as the time from the start of bevacizumab treatment until clinical progression as determined by the treating neuro-oncologist, and/or radiographic progression determined by RANO criteria [23]. Patients who stopped bevacizumab treatment for reasons other than progression were censored. In addition overall survival and time on bevacizumab were assessed. Bevacizumab was discontinued due to progression, toxicity or complications as determined by the individual treating neurooncologist. Since the determination of clinical progression is often based on MRI progression, we analyzed MRI changes during bevacizumab treatment. It is well known that bevacizumab can diminish gadolinium enhancement after contrast [24], so we included flair changes and multifocality changes in addition to contrast enhancement [23]. Radiographic progression while on bevacizumab was noted as either an increase in tumor volume on FLAIR or T1 weighed images with contrast or as the development of an additional lesion(s) (multifocal disease) on post treatment MRI, compared to pretreatment MRI. We included both clinical and radiographic progression in our outcomes evaluation, because clinical deterioration in this patient population is often attributed to disease progression in the absence of clear etiology such as seizures, even though it is not always extensively investigated. Other reasons for deterioration such as cognitive decline related to previous therapy (e.g. radiation), side effects of other medications or delirium, can therefore not be fully excluded.

Multifocal change

Since a more invasive behavior of GBM has been described in the setting of treatment with bevacizumab, we specifically looked at multifocal change [13]. We defined multifocal change as the appearance of a discrete new lesion (on FLAIR or T1 + contrast sequences), which have no visible connection to the existing lesion.

Volumetric analysis

Volumetric measurements were obtained using iPlan Net 3.0.0 software (BrainLAB AG, Germany 2009). Regions of interest (ROIs) were manually drawn by two neurosurgeons (KEH and YE) on each post-contrast axial T1-weighted and FLAIR image and used to compute the volume of the tumor in cubic centimeters. Tumor volume was measured on the MRI scan nearest to the start date and end point of bevacizumab treatment.

Statistical methods

Statistical testing was designed and performed by statisticians (JZ and KP). Associations between patient characteristics and MRI features with genomic subtype and molecular profile were examined using Fisher's exact test and the Kruskal–Wallis test. Time on bevacizumab was compared between

genomic subtypes using Kruskal–Wallis test since all patients stopped using bevacizumab at our last follow-up. The Kaplan–Meier method and Cox proportional hazards regression models were fit to evaluate associations of genomic subtype, age, gender, KPS, molecular profiles with time to progression on bevacizumab and overall survival (OS). Death was not a competing risk in the analysis of progression considering that all deaths occurred after stopping bevacizumab. Considering the number of patients who had undetermined EGFR amplification (19%) or EGFR VIII expression (48%), undetermined status were treated as a separate group in above analyses.

A p value less than 0.05 was considered statistically significant. All analyses were performed in software packages SAS 9.4 (SAS Institute Inc., Cary, NC, USA), and R version 3.1 (The R Foundation for Statistical Computing).

Results

Patient characteristics

This study identified 84 glioblastoma patients with transcriptomally-defined TCGA subtype who received bevacizumab for recurrent tumor. Four patients were excluded due to insufficient documentation of treatment and MRI dates. Table 1 describes characteristics of the 80 patients: 21% classical, 29% mesenchymal, 25% proneural and 5% neural. Clinical or radiographic progression was found in 65 patients. In 15 patients bevacizumab was stopped for reasons other than clinical or radiographic progression. Those included: wound dehiscence, nephrotoxicity, DVT, bowel perforation and intracranial hemorrhage. Among those patients, 8 had a mesenchymal subtype, 3 had a classical, 2 had a proneural and 2 had a neural subtype. Age and KPS were similarly distributed across the different genomic subtypes. The median time on bevacizumab was 4.6 months (range 0.2–32.2). The pre-treatment MRI was performed within 66 days (interquartile range (IQR) 6–16) prior to bevacizumab start, and post-treatment MRI was performed between 75 days prior to bevacizumab discontinuation and 209 days post bevacizumab end (IQR 2 prior–3 post). Although this is a wide range, most were done within a few days of treatment start and discontinuation; and time of MRI did not differ significantly among

Table 1 Patient, molecular and imaging characteristics by TCGA subtype (N = 80)

	All patients		Classical	Mesenchymal		Neural	Proneural	p value
N	80		21	29		5	25	
	Median (range)		Median (range)	Median (range)		Median (range)	Median (range)	
Age at treatment	60.4 (25.1, 80.33)		58.2 (40.1, 77.1)	61.5 (34.4, 80.3)		65.5 (45.6, 71.5)	59.6 (25.1, 74.1)	0.782
Pre-treatment volume on MRI, cm ³	20.03 (0, 96.36), n=75		14.3 (0.5, 48.5)	26.5 (0, 96.4)		25.6 (6, 91.7)	15.9 (0.7, 89)	0.255
Post treatment volume on MRI, cm ³	17.59 (0, 155.56), n=72		25.4 (0, 155.6)	19.7 (0.2, 149.1)		22.9 (3.1, 91.7)	13.2 (0, 97)	0.457
% Volume change on MRI	1% (- 100%, 4484%), n = 72		120% (- 4484%)	12% (- 98%, 2088%)		- 66% (- 1436%)	- 26% (- 1283%)	0.272
Pre-treatment volume on MRI flair, cm ³	109.21 (2.66, 275.65), n=73		101.1 (6.2, 235.3)	119.9 (4.9, 275.6)		52.2 (9.8, 275.3)	88.7 (2.7, 229.2)	0.513
Post treatment volume on MRI flair, cm ³	102.32 (22.73, 283.25), n = 70		68.1 (27.5, 278.3)	114.9 (33.4, 283.2)		104.8 (72.8, 173.5)	96 (22.7, 210.9)	0.165
% Volume change on MRI flair	- 2% (- 74%, 1902%), n = 70		- 4% (- 69%, 1902%)	2% (- 72%, 306%)		133% (- 51%, 639%)	- 13% (- 1396%)	0.595
Time to treatment failure ^a , months	4.61 (0.23, 32.2)		2.7 (0.9, 10.1)	5.1 (0.2, 32.2)		6.4 (3.7, 15.2)	6 (0.5, 14.5)	0.011

	N(%)	N(%)	N(%)	N(%)	N(%)	
Gender						0.677
F	33 (41%)	11 (33%)	11 (33%)	2 (6%)	9 (27%)	
M	47 (59%)	10 (21%)	18 (38%)	3 (6%)	16 (34%)	
Multifocal change						0.690
N	69 (92%)	17 (25%)	26 (38%)	4 (6%)	22 (32%)	
Y	6 (8%)	2 (33%)	1 (17%)	0 (0%)	3 (50%)	
KPS						0.480
50–70	36 (45%)	12 (33%)	13 (36%)	1 (3%)	10 (28%)	
80–100	44 (55%)	9 (20%)	16 (36%)	4 (9%)	15 (34%)	
MGMT methylated status						0.011
No	41 (51%)	7 (17%)	18 (44%)	2 (5%)	14 (34%)	
Yes	14 (18%)	6 (43%)	0 (0%)	2 (14%)	6 (43%)	
Unknown	25 (31%)	8 (32%)	11 (44%)	1 (4%)	5 (20%)	
EGFR gene amplified						< 0.001
No	37 (46%)	0 (0%)	17 (46%)	2 (5%)	18 (49%)	
Yes	28 (35%)	16 (57%)	5 (18%)	3 (11%)	4 (14%)	
Unknown	15 (19%)	5 (33%)	7 (47%)	0 (0%)	3 (20%)	
EGFR VIII status						0.207
No	30 (38%)	8 (27%)	10 (33%)	0 (0%)	12 (40%)	
Yes	12 (15%)	4 (33%)	2 (17%)	2 (17%)	4 (33%)	
Unknown	38 (48%)	9 (24%)	17 (45%)	3 (8%)	9 (24%)	

KPS Karnofsky performance score, MGMT O⁶-methylguanin-DNA-methyltransferase, EGFR epidermal growth factor receptor

^aAll patients were off Avastin at the last followup

the different subtypes ($p=0.783$). The majority of patients who progressed received other therapies during and after stopping treatment with bevacizumab, which were not statistically different amongst the different subtypes (Supplementary Table S1).

Fig. 1 Time to progression on bevacizumab by **a** TCGA subtype **b** EGFR amplification status. Kaplan–Meier curve for time to progression on bevacizumab stratified by TCGA subtype (**a**) and EGFR amplification status (**b**)

Table 2 Time to progression on bevacizumab (N = 80)

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p value	HR (95% CI)	p value
Age at treatment start	1.00 (0.97, 1.02)	0.829		
Baseline flair MRI volumea, cm ³	1.01 (0.97, 1.04)	0.742		
Gender		0.854		
F	1			
M	1.05 (0.64, 1.73)			
Nanostring		< 0.001		0.010
Classical	1		1	
Mesenchymal	0.26 (0.13, 0.52)		0.39 (0.17, 0.88)	
Neural	0.22 (0.06, 0.77)		0.23 (0.06, 0.85)	
Proneural	0.43 (0.23, 0.82)		0.76 (0.29, 1.95)	
KPS		0.805		
50–70	1			
80–100	1.07 (0.64, 1.76)			
MGMT methylated status		0.725		
No	1			
Yes	1.29 (0.66, 2.53)			
Unknown	1.16 (0.66, 2.04)			
EGFR gene amplified		0.007		0.010
No	1		1	
Yes	2.39 (1.36, 4.18)		4.00 (1.63, 9.77)	
Unknown	1.32 (0.67, 2.61)		2.25 (1.03, 4.92)	
EGRF VIII status		0.014		0.001
Absence	1		1	
Presence	0.67 (0.34, 1.32)		0.35 (0.16, 0.79)	
Unknown	0.44 (0.25, 0.78)		0.33 (0.17, 0.62)	

HR hazard ratio

Progression on bevacizumab was defined as either clinical or radiologic progression (N = 65)

^aThe increment of hazard ratio estimates is every 10-unit increase in these size measurements

Classical glioblastomas have the shortest time on bevacizumab and a higher risk of progression

Time on bevacizumab was significantly shorter for the classical GBMs compared to the mesenchymal, neural and proneural subgroups (2.7 vs. 5.1, 6.4 and 6.0 months respectively, p= 0.011, Table 1). The classical subgroup also had a higher risk of progression than other subgroups in univariate analysis (p < 0.001, Fig. 1a; Table 2) and remained significant in multivariate analysis

Fig. 2 Overall survival (OS) by **a** TCGA subtype and **b** EGFR amplification status. Kaplan–Meier survival curve stratified by TCGA subtype (**a**) and EGFR amplification status (**b**)

($p = 0.010$). Of note, in the mesenchymal group 3 patients (10.3%) remained on bevacizumab 18 months or more versus none in the proneural and classical groups, suggesting some durability to this treatment in a select subgroup of patients.

EGFR amplification is associated with a shorter time to progression

EGFR amplification was determined in 85% of the patients and in 43% (28/65) of them the gene was amplified. As expected, the amplified *EGFR* gene was significantly more often present in the

classical subtype tumor ($p < 0.001$). Interestingly, amplified EGFR status was associated with a higher risk of progression on bevacizumab ($p = 0.007$, Fig. 1b; Table 2). This finding remained significant after controlling for genomic subtype and EGFRV8 expression in multivariate analysis ($p = 0.010$, Table 2). *MGMT* promoter methylation status was available in 69% of the patients and was more often unmethylated in patients with mesenchymal tumors ($p = 0.011$) but was not associated with a risk of progression ($p = 0.725$, Tables 1, 2).

Overall survival by EGFR amplification status and subtype

All but 1 of the patients in this cohort died. The median overall survival from treatment initiation was 7.9 months (95% CI 6.5–11.4). Consistent with time to progression, patients with either the classical phenotype or EGFR-amplified tumors seemed to do worse although these differences were not statistically significant (Fig. 2a, b). These results should be interpreted with caution in light of the additional treatments that these patients received.

No significant difference in tumor volume and multifocal change between subtypes

Large differences in tumor volume and in volume changes were noted in the different subtypes on T1 with contrast and FLAIR MRI images, but none reached statistical significance (Table 1). Multifocal change while under bevacizumab treatment was a relatively common finding (92%) but it was not different among the subtypes (Table 1).

Discussion

Our data suggest that glioblastomas that exhibit EGFR amplification or classical TCGA subtype are associated with a shorter time to progression on bevacizumab in the recurrent setting, in comparison to tumors with a proneural, neural and mesenchymal subtype or tumors without EGFR amplification. Patients with a classical subtype had a median time to progression of 2.8 months (95% CI 1.6–4.8) and in patients with EGFR amplified tumor, the median time to progression was 3.7 months (95% CI 1.6–5.6). A worse outcome in EGFR-amplified and classical tumors was statistically significant on both univariate and multivariate analysis. No difference in tumor volume change or multifocal change while on bevacizumab was observed among the different tumor subtypes. Our results therefore suggest that this treatment may be better reserved for patients without EGFR amplification or with a non-classical subtype.

This study has several limitations: the two most significant being the retrospective nature of the analysis and the different and intensive treatments that patients received in addition to bevacizumab. Even though receiving additional treatments was similarly distributed across the tumor subtypes, a treatment-related potential bias cannot be excluded. Another limitation is the fact that the decision

to discontinue bevacizumab was often made on clinical grounds, as determined by the treating neuro-oncologist, and not necessarily always with clear evidence for radiographic progression. However the high volume of glioblastoma patients treated at MSKCC and the fact that treatment decisions are made in a multidisciplinary setting (e.g. tumor board) increases the uniformity of decision making across patients. Strengths of the study include the objective nature of the radiographic analysis and the fact that the treating physicians were blinded to the tumor subtype at the time of treatment. Another strength of the study is that the tissue analysis was done on the recurrent tumor. This is especially important since there are many genetic and epigenetic differences between the primary neoplasm at diagnosis and the recurrent tumor [25]. An important recent initiative to understand this process better is the Glioma Longitudinal Analysis (GLASS) Consortium, which has been initiated to better understand the recurrence process and, in doing so, discover vulnerabilities that can be used for therapeutic intervention [26].

Our finding of a strong association between EGFR amplification and a shorter time to progression on bevacizumab is novel. The fact that EGFR amplification alone also resulted in a statistically powerful difference in terms of progression on bevacizumab is important, as it is more readily determined by a simple widely available test (FISH analysis). TCGA class analysis might not be routinely available in every institution. Also, EGFR amplification is usually retained from primary to recurrent tumors [27]. It is interesting to note that some patients with mesenchymal tumors, that were all MGMT unmethylated, seemed to do better on bevacizumab in this study. In a few cases some patients managed to stay on this treatment for more than 18 months, suggesting that the mesenchymal subtype may be more suitable to this treatment. This is especially promising since this subtype was shown to have the worst prognosis and recurrent tumors have often shown a switch to this subtype [28]. Although the mesenchymal subtype has the most “angiogenic” signature, thus was expected to respond most to bevacizumab, a recent report suggested that the proneural subclass benefited more from bevacizumab treatment in the upfront setting. In a retrospective analysis of the AvAglio trial, patients with wild type IDH proneural glioblastomas had a significant overall survival advantage of 17.1 versus 12.8 months versus placebo. The underlying mechanism of this preferential response is still unclear [29]. Interestingly, another phase II trial, that looked at hypofractionated stereotactic radiotherapy schedule with temozolomide and bevacizumab for newly diagnosed glioblastomas, found that proneural tumors did worse than the other subtypes, although this did not reach statistical significance for overall survival [21]. As mentioned earlier, molecular analysis of patients in the BELOB trial led the authors to conclude that in the recurrent setting classical tumors had the longest progression free survival when treated with bevacizumab and CCNU [20]. Even though this patient cohort was much more uniform, the tissue analysis was done on the original tumor and not in the recurrent setting as in our study which is known to change [25]. By far the most tumors (68%) in the BELOB trial were classical and other types were grouped as “non-classical” because of low numbers, which may have impacted the results. Also their findings were only in the combination treatment group and not bevacizumab alone. The finding of mesenchymal tumors responding for an extended period of time to bevacizumab in the recurrent setting has not been reported previously, to our knowledge. It has become evident in recent years that bevacizumab

treatment is not without risk, in particular from wound breakdown and the possibility of inducing a more infiltrative/aggressive tumor after treatment [14]. Our analysis suggests that bevacizumab should be considered more carefully in classical and EGFR-amplified tumors and may be more beneficial for mesenchymal, neural and proneural tumors. Nevertheless bevacizumab remains one of the few options available to patients with recurrent tumors that have failed standard of care and available trials. Neuro-oncologists have thus maintained this drug in their armamentarium, especially since it also offers a significant anti-edema activity. It is therefore highly relevant to investigate potential predictors of high failure risk. Further work will be needed to identify a potential mechanistic antagonism in the context of EGFR amplification that might explain these results. Alternatively, confounding factors such as the stage at which bevacizumab is used in these tumors, specific prior or concomitant treatment regimens may contribute to the worse results seen in this group.

Conclusion

Classical subtype and EGFR-amplified glioblastomas show a worse response to bevacizumab in the recurrent setting. Additional investigations are required to further validate this finding as well as to investigate the mechanistic basis for bevacizumab propensity to fail in the setting of amplified EGFR.

Compliance with ethical standards

Conflict of interest All the authors declare that there is no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent The research protocol was submitted to the institutional research board and deemed exempt. No patient consent was required.

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Supplementary table S1: additional treatment with avastin stratified by (a) TCGA subtype and (b) EGFR amplification status

	Classical	Mesenchymal	Proneural	p Value
	n (%)	n (%)	n (%)	
Number of treatment pre bevacizumab				
0	1 (5%)	1 (5%)	0 (0%)	0.453
1	5 (26%)	5 (24%)	5 (22%)	
2	7 (37%)	9 (43%)	10 (43%)	
3	4 (21%)	6 (29%)	3 (13%)	
4	2 (11%)	0 (0%)	5 (22%)	
Number of treatment during bevacizumab				
0	11 (58%)	9 (43%)	9 (39%)	0.588
1	5 (26%)	10 (48%)	10 (43%)	
2	2 (11%)	2 (10%)	4 (17%)	
3	1 (5%)	0 (0%)	0 (0%)	
Number of treatment post bevacizumab				
0	10 (53%)	13 (62%)	17 (74%)	0.133
1	5 (26%)	7 (33%)	3 (13%)	
2	4 (21%)	0 (0%)	1 (4%)	
3	0 (0%)	1 (5%)	1 (4%)	
4	0 (0%)	0 (0%)	1 (4%)	

EGFR amplification status				
	No	Yes	Unknown	p value
Number of treatment pre bevacizumab				
0	0 (0%)	2 (9%)	0 (0%)	0,298
1	8 (30%)	6 (26%)	1 (8%)	
2	10 (37%)	7 (30%)	9 (69%)	
3	5 (19%)	5 (22%)	3 (23%)	
4	4 (15%)	3 (13%)	0 (0%)	
Number of treatment during bevacizumab				
0	11 (41%)	11 (48%)	7 (54%)	0,785
1	13 (48%)	7 (30%)	5 (38%)	
2	3 (11%)	4 (17%)	1 (8%)	
3	0 (0%)	1 (4%)	0 (0%)	
Number of treatment post bevacizumab				
0	20 (74%)	15 (65%)	5 (38%)	0,109
1	5 (19%)	5 (22%)	5 (38%)	
2	1 (4%)	3 (13%)	1 (8%)	
3	0 (0%)	0 (0%)	2 (15%)	
4	1 (4%)	0 (0%)	0 (0%)	

Chapter 4

4

Glioblastoma stem-like cells give rise to tumour endothelium

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Glioblastoma (GBM) is among the most aggressive of human cancers¹. A key feature of GBMs is the extensive network of abnormal vasculature characterized by glomeruloid structures and endothelial hyperplasia². Yet the mechanisms of angiogenesis and the origin of tumour endothelial cells remain poorly defined³⁻⁵. Here we demonstrate that a subpopulation of endothelial cells within glioblastomas harbour the same somatic mutations identified within tumour cells, such as amplification of *EGFR* and chromosome 7. We additionally demonstrate that the stem-cell-like CD133⁺ fraction includes a subset of vascular endothelial-cadherin (CD144)-expressing cells that show characteristics of endothelial progenitors capable of maturation into endothelial cells. Extensive *in vitro* and *in vivo* lineage analyses, including single cell clonal studies, further show that a subpopulation of the CD133⁺ stem-like cell fraction is multipotent and capable of differentiation along tumour and endothelial lineages, possibly via an intermediate CD133⁺/CD144⁺ progenitor cell. The findings are supported by genetic studies of specific exons selected from The Cancer Genome Atlas⁶, quantitative FISH and comparative genomic hybridization data that demonstrate identical genomic profiles in the CD133⁺ tumour cells, their endothelial progenitor derivatives and mature endothelium. Exposure to the clinical anti-angiogenesis agent bevacizumab⁷ or to a γ -secretase inhibitor⁸ as well as knockdown shRNA studies demonstrate that blocking VEGF or silencing *VEGFR2* inhibits the maturation of tumour endothelial progenitors into endothelium but not the differentiation of CD133⁺ cells into endothelial progenitors, whereas γ -secretase inhibition or *NOTCH1* silencing blocks the transition into endothelial progenitors. These data may provide new perspectives on the mechanisms of failure of anti-angiogenesis inhibitors currently in use. The lineage plasticity and capacity to generate tumour vasculature of the putative cancer stem cells within glioblastoma are novel findings that provide new insight into the biology of gliomas and the definition of cancer stemness, as well as the mechanisms of tumour neo-angiogenesis.

Blood vessels within GBM express a variety of markers, including CD31 and CD105 (also known as PECAM1 and ENG, respectively); CD105 is a proliferation-associated molecule expressed in angiogenic endothelium⁹. Quantitative analysis of 16 GBM specimens by fluorescence-activated cell sorting (FACS) and immunohistochemistry showed that more than 70% of CD105⁺ cells co-express CD31 (Fig. 1a, b), VEGFR2 (also known as KDR) and von Willebrand factor (also known as VWF), exhibit endothelial morphology, and labelling by DiI-AcLDL (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-in docarbocyanine perchlorate-labelled acetylated low density lipoproteins, ref. 10), suggesting an endothelial phenotype (Supplementary Fig. 1a). On average, 5% of the total cell population expressed CD31 in normal brain and GBM specimens ($n = 7$), whereas CD105⁺ cells were essentially absent in normal brain (Supplementary Fig. 1b). CD105⁺ cells were also isolated by FACS from fresh GBM specimens and injected with a collagen matrix¹¹ into the flank of NOD-SCID mice. The resulting implants were composed of a network of vascular channels of human origin, expressed CD105 and CD31 and showed evidence of uptake of systemically injected lectin (Fig. 1c).

Whereas endothelial cells in GBMs are often classified as “hyperplastic”², the abnormal blood vessel architecture, the distinct gene expression profiles¹² and the selective emergence of abnormal vessels in GBMs versus lower grade gliomas² suggest a more complex ontogeny of GBM endothelium. We performed quantitative fluorescence *in situ* hybridization (FISH) analyses for *EGFR* and chromosome 7 (ref. 13) on CD105⁺ cells isolated by FACS and on sections of the corresponding GBM parent tumour (Fig. 1d, e and Supplementary Fig. 2). The proportion of CD105⁺ cells harbouring 3 copies of the *EGFR* amplicon or the centromeric portion of chromosome 7 was comparable to the proportion of tumour cells with the same aberrations (Supplementary Table 1a). We also performed quantitative PCR (qPCR) for three segments of the *EGFR* amplicon (exons 4, 9 and 11), known to be mutated at high frequency according to data from The Cancer Genome Atlas⁶. The data demonstrate a similar copy number in the CD105⁺ cells and the corresponding parent tumour (Supplementary Table 1b) and indicate that a proportion of tumour endothelial cells within GBM is in fact neoplastic. CD133 is a cell surface glycoprotein used extensively as a marker of putative cancer stem cells (CSCs) but also expressed in haematopoietic stem cells^{14–18}. Although the specific identity and definition of CSCs remains a matter of debate, we proposed that the CD133⁺ fraction may be related to the endothelial differentiation potential observed. Acutely dissociated cells from a series of 14 GBMs were fractionated into four groups: (1) CD144⁺/CD133⁺, (2) CD144⁺/CD133⁺ (double positive, DP), (3) CD133⁺/CD144⁺ and (4) CD133⁺/CD144⁺ (double negative, DN) (Fig. 2a). All samples contained the four fractions, with the DN being the largest population (Supplementary Table 4). Quantitative PCR with reverse transcription (qRT-PCR) analysis for endothelial markers (Supplementary Fig. 3a) demonstrated marked enrichment of *VEGFR2* and the endothelial progenitor marker *CD34* in the CD144⁺/CD133⁺ and in the DP populations. CD105 was consistently absent in the CD133⁺ and CD144⁺ fractions. To define lineage potential further, DP cells were cultured for 5 days in endothelial cell medium which resulted in the downregulation of CD144, the upregulation of CD105 and CD31 as well co-expression of VEGFR2 and CD34 and labelling

Figure 1 | CD105⁺ endothelial cells in GBM harbour genomic aberrations. a, FACS analysis and quantification of GBM-derived CD105⁺ cells shows co-expression of other endothelial cell markers (CD31, VEGFR2) and uptake of DiI-AcLDL (*n* 53). FITC, fluorescein isothiocyanate; PE, phycoerythrin. b, CD105 immunostaining in GBMs delineates microvessels co-labelling with CD31 and glomeruloid vessels surrounded by caldesmon (CALD)-expressing pericytes. c, Functional neovessel formation by GBM-derived CD105⁺ cells in the flank of NOD-SCID mice. Confocal immunofluorescence demonstrates co-localization of a human mitochondria marker with CD31 and uptake of lectin by the CD105⁺ vessels in the implants. d, Immuno-FISH of CD105⁺ vessels in GBM specimens (case 76, 78) shows multiple copies of the *EGFR* amplicon (arrows). e, FISH on CD105⁺ cells sorted from GBMs confirms amplification of *EGFR* (red) and chromosome 7 centromere (Chr7, green) (arrows). Control nuclei, individually contoured, are from normal human fibroblasts. Scale bars, 50 μ m. Error bars, s.d.

Figure 2 | GBM-derived CD133⁺ cells include a fraction of endothelial progenitors a, Representative FACS analysis of a GBM specimen with fractionation into four cell subpopulations based on the expression of CD133 and vascular E-cadherin (CD144). b, Immunofluorescence analysis of DP (CD133⁺/CD144⁺) cells upon differentiation demonstrates co-expression of endothelial markers and DiI-AcLDL uptake. c, d, In Matrigel, DP cells will exhibit DiI-AcLDL uptake and form tubular networks comparable to those shown by normal endothelial cells, as well as areas of thickened walls where cells are more proliferative. Scale bars, 100 μ m in b and d; 300 μ m in c.

Figure 3 | CD133⁺/CD144⁺ cells are multipotential and give rise to endothelial cells via an endothelial progenitor intermediate. a, b, Co-cultures of CD133⁺/CD144⁺ cells with tumour cells give rise to endothelial progenitors that co-express CD133 and CD144 (DP) as shown and quantified by FACS analysis ($n = 3$). APC, allophycocyanin. c, GFP⁺-derived DP cells form intracellular vacuolar structures in collagen gel, characteristic of endothelial cells. d, Immunohistochemistry of CD133⁺/CD144⁺-derived

endothelial cells ($n = 3$). e, f, Single cell clonal analysis of GFP-labelled CD133⁺/CD144⁺ cells. GFP⁺ clones derived from single cells are seeded under neural or endothelial conditions. Normal endothelial precursor cultures (EPC) and human dermal fibroblasts (HDF) were used as controls. Under endothelial conditions, all cells except HDF express endothelial but not neural markers. Under neural conditions, cells from the same GFP/CD133⁺ clone are positive for GFAP and nestin but not endothelial markers, while controls are negative for all markers. Scale bar, 50 μ m. Errors are s.d.

with DiI-AcLDL (Fig. 2b and Supplementary Fig. 3b). When grown in three-dimensional (3D) gel cultures, the *in vitro* DP- derived endothelial cells form vascular networks reminiscent of normal endothelium, but also thickened channel walls and areas of confluence more suggestive of abnormal tumour vessels (Fig. 2c, d). The primary CD105⁺ cells also form glomeruloid-like structures in 3D gel, with high lectin uptake (Supplementary Fig. 1c). DP-derived CD105⁺ cells were sorted and injected subcutaneously in NOD/SCID mice, giving rise to vascularized plugs identical to those obtained from primary CD105⁺ cells (Supplementary Fig. 1d).

The CD144⁺/CD133⁺ cell fraction was often very small but showed a restricted differentiation and immunohistochemical profile (Supplementary Fig. 3c, d). When grown in Matrigel, the CD144⁺/CD133⁺ cells develop tubular, capillary-like structures¹⁹ and nogglomeruli (Supplementary Fig. 3e). CD144⁺/CD133⁺ cells do not express neural markers or form neurospheres, thus indicating a more restricted endothelial precursor cell identity (Supplementary Fig. 3f). Unsupervised clustering of transcriptome data was performed on several data sets including independent samples of the four sorted tumour subpopulations, as well as CD144⁺ human embryonic stem-cell-derived endothelial precursors and bone-marrow-derived CD34⁺ endothelial progenitors (Supplementary Fig. 3g). Taken together, these results indicate that GBMs comprise cell fractions capable of endothelial cell differentiation.

The identification of genomic aberrations in tumour endothelium and the presence of endothelial progenitors within the CD133⁺ putative CSC fraction in GBMs, led us to postulate that DP cells may represent the neoplastic origin of tumour endothelium and could derive from the CD133⁺ CSC fraction. CD133⁺/CD144⁺ cells were then labelled with *EFa-1::GFP* (elongation factor a1–green fluorescent protein) lentiviral vectors, triple sorted, and GFP⁺/CD133⁺/CD144⁺ cells were cocultured in the presence of tumour cells. On day 5, FACS analysis demonstrated the emergence of a GFP⁺-DP population (Fig. 3a, b). When placed in collagen cultures, the GFP⁺-DP cells had intracellular vacuoles suggestive of early lumen formation by endothelial tubes²⁰ (Fig. 3c), and differentiation into cells that express CD105 and CD31 and exhibit DiI-AcLDL uptake (Fig. 3d and Supplementary Fig. 4a). Importantly, co-culture with tumour cells is essential for the emergence of DP cells (Fig. 3a, b). Our data confirm that the DP endothelial progenitors within GBM can arise from the CD133⁺ cell population and are capable of differentiating into endothelial cells of tumour origin. Of note, the tumour cells used in these co-culture experiments originate from tumours with different genetic backgrounds and transcriptomal subclasses (Supplementary Tables 2 and 3).

Recent data support a close interaction²¹ or a lineage relationship²² between endothelial cells and neural stem cells. We next explored whether endothelial differentiation of CD133⁺/CD144⁺ can be further promoted by extrinsic signals. To this end, CD133⁺/CD144⁺ cells were isolated from GBM samples, stably transduced with *EFa-1::GFP* lentiviral vectors, sorted for GFP⁺/CD133⁺/CD144⁺ and co-cultured with tumour-derived endothelial cells. GFP-expressing endothelial cells were identified at 7–10 days *in vitro* as demonstrated by co-labelling of GFP with CD105 and CD31, and also incorporation of DiI-AcLDL. Control experiments using GFP-labelled CD133⁺ cells did not yield any endothelial cells (Supplementary Fig. 4b). The CD133⁺/CD144⁺

population formed neurospheres and readily differentiated along the three main CNS lineages (Supplementary Fig. 4c). Whereas these data are suggestive of the multipotent nature of the CD133¹/CD144² cells, they do not rule out the presence of heterogeneous populations within the CD133¹/CD144² fraction with distinct differentiation potentials. We thus performed single-cell clonal studies of CD133¹/CD144² cells as well as normal endothelial cells and fibroblasts as controls (Supplementary Fig. 4d). The data demonstrate both endothelial and neural differentiation potential within a single-cell derived clone confirming that CD133¹/CD144² cells are capable of generating tumour cells and tumour-derived endothelium (Fig. 3e, f). FISH for *EGFR* and chromosome 7 in the clones confirmed the presence of genomic amplifications identical to those exhibited by the parent tumour tissue (Supplementary Fig. 4e).

We next tested the fate of the various tumour cell fractions upon transplantation *in vivo*. CD133¹/CD144², DP, CD144¹/CD133² and DN cells were injected into the striatum of immunodeficient mice. All grafted animals developed tumours with the exception of those that CD133¹/CD144² gave rise to large, highly infiltrative and hyperproliferative tumours showing strong expression of nestin (Fig. 4a). Whereas all xenograft tumours had a comparable volume and proliferation rate, the DP-derived tumours showed significantly increased levels of vascularization as demonstrated quantitatively (Supplementary Fig. 5a).

Some of the animals were grafted with stably GFP-marked CD133¹ cells allowing us to serially passage GFP-labelled CD133¹/CD144² cells from the primary xenograft in NOD-SCID mice. Secondary tumours formed at similar efficiency and showed comparable cell composition to the first passage cells. FACS analysis of GFP labelled xenograft cells demonstrates expression of endothelial markers, including CD105 and CD34 (Fig. 4b). After a second passage *in vivo*, tumours were sorted again for GFP¹/CD133¹/CD144² cells, which upon culture gave rise to GFP-labelled CD31¹ and CD105¹ cells, thus demonstrating maintenance of the multipotential phenotype (Fig. 4c). Immunohistochemical analysis, including confocal microscopy, demonstrated tumour blood vessels with typical morphology that express human markers. Tumour-bearing animals were also injected systemically with lectin, resulting in vessel-specific uptake and colabelling with human markers (Fig. 4d, e and Supplementary Fig. 6). Thus, multipotency—including differentiation capacity along endothelial lineages—is maintained within the CD133¹/CD144² population *in vivo* and upon passaging. However, in the absence of clonal studies *in vivo*, true multipotency of tumour stem-like cells cannot be definitively confirmed.

A more comprehensive and quantitative analysis of genomic aberrations was conducted in order to verify the lineage relationship among the different tumour subpopulations. qPCR for the *EGFR* exons as described above⁶ demonstrates the highest copy number within the CD133¹/CD144² population followed by the endothelial progenitors (DP) and the CD105¹ cells (Supplementary Fig. 5b). Interestingly, the CD31¹ cells and the CD144¹/CD133² progenitors showed lower levels of amplification, indicating that they may include a significant proportion of genotypically normal cells. We propose that these cells largely represent normal endothelium and circulating endothelial progenitors, respectively. This is compatible with the more restricted endothelial fate demonstrated by the CD144¹/CD133² cells as shown above (Supplementary

Figure 4 | Cancer stem-like cells and endothelial progenitors give rise to tumour and endothelial cells *in vivo*. a, Representative magnetic resonance imaging (MRI) images from mice that received injection of DN, CD133⁺/CD144⁻ or DP cells from primary GBM specimens. T2 sequences demonstrate infiltrative tumours except in the DN group. Tumours were hypercellular on haematoxylin and eosin (H&E), showed high proliferation rates (Ki67) and nestin expression. Immunostaining for human-specific CD31 demonstrates the presence of vessels of human origin within the tumours. NA, human nuclear antigen. b, FACS plots (left) and quantitative analysis (right) for endothelial marker expression in xenograft tumours (GFP⁺/CD133⁺/CD144⁻ cells) and controls (DN). (*n* 5 3, s.d.). 7-AAD, 7-aminoactinomycin. FL-1 and 2, fluorescent channels 1 and 2; mIgG, mouse immunoglobulin G. c, Xenograft derived GFP⁺/CD133⁺/CD144⁻ cells express endothelial markers upon *in vitro* differentiation (arrows). d, Uptake of systemic lectin in tumour xenografts demonstrates blood vessels that co-label with human endothelial markers (CD31 and CD105). e, Confocal microscopy of xenograft microvasculature. Scale bars, 100 mm in a; 50 mm in c; 140 mm in d; 10 mm in e.

Fig. 3c, e, f). Quantitative FISH studies for copy number of *EGFR* and chromosome 7 per cell were performed on CD133¹/CD144², DP and CD105¹ cells and revealed a substantial proportion of cells bearing the neoplastic aberrations in each population, ranging from 47.3% to 71.7% (Supplementary Fig. 5c). To address genomic alterations in tumour cells in a more unbiased manner we performed array comparative genomic hybridization (CGH) on the fractionated populations (Supplementary Fig. 7). The CGH data showed similar patterns of genomic aberrations in tumour cells as well as the endothelium and its progenitors, at variable amplitudes and across different regions, thus demonstrating a similar paradigm even in tumours that do not exhibit *EGFR* gain. We performed transcriptome analyses on a set of 18 tumours used in this study and found a random distribution of commonly described genotypes as well as representation of all TCGA-defined transcriptomal classes (Supplementary Table 3). Finally, we performed metaphase spreads on purified cell fractions of CD133¹/CD144², DP and CD105¹ following short-term culture. The majority of the cells had a highly abnormal but neardiploid karyotype, indicating that nuclear fusion is a very unlikely explanation for the lineage transition from cancer cell to endothelial progenitor or mature cell (Supplementary Fig. 5d). Vascular mimicry has been described in melanoma²³ and other tumours²⁴; aneuploidy was also shown in renal cell cancer endothelium, but not matched to parent tumour cells²⁵.

We investigated the impact of DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester), a γ -secretase inhibitor that effectively inhibits Notch signalling⁸, and bevacizumab, a VEGFA-binding antibody⁷ currently in clinical use, on the differentiation of CD133¹/CD144² to DP and then to CD105¹ cells. Exposure to bevacizumab did not have an impact on the ability of CD133¹/CD144² cells to differentiate into endothelial progenitors, yet it blocked further maturation from DP into CD105¹ endothelial cells. In contrast, γ -secretase inhibition resulted in significant suppression of the transition from CD133¹/CD144² to DP, but did not affect maturation to CD105¹ cells. To demonstrate the specific roles of the Notch and VEGF pathways, we performed knockdown studies targeting the *NOTCH1* and *VEGFR2* receptors. The gene silencing data further supported the results of the inhibitor studies (Supplementary Figs 8b and 9). Gene expression analysis shows significant upregulation of *NOTCH1/2* and *VEGFR1/2* in the CD133¹/CD144² and DP groups, respectively (Supplementary Fig. 8). These preliminary studies offer a novel perspective of the roles of the VEGF and Notch pathways in glioma biology, although the functional consequences of VEGF or Notch blockade remain to be determined.

Despite some promise, bevacizumab therapy is often interrupted by GBM progression characterized by a decrease in abnormal vascularity and significant invasive tumour behaviour²⁶. Based on the paradigm presented here (Supplementary Fig. 9a), bevacizumab failure could be conceivably due to the disruption of the dynamic relationships between the tumour fractions.

In summary, our data demonstrate that a subpopulation of cells within GBM can give rise to endothelial cells via a bipotential progenitor intermediate, and that the CD133¹ cancer stem-cell-like fraction includes a population of endothelial progenitors. An in-depth understanding of the lineage relationship between tumour cells and endothelial progeny should provide new insights into CSC biology and tumour self-renewal. Given the strong correlation of tumour

grade and neoplastic vasculature in human gliomas, agents that could block endothelial transition of tumour cells may provide a novel therapeutic strategy for this currently intractable disease.

Methods summary

All experiments were conducted on freshly obtained surgical specimens of glioblastoma tumour; a neuropathologist confirmed the diagnosis on frozen section before tissue acquisition. Tumours were newly diagnosed or recurrent. A total of 78 tumours were used in the study. Cell fractions were sorted using standard methods at our FACS facility; *in vitro* experiments were conducted on shortpassage cultures (maximum of five passages) if needed. A total of 34 xenografts were obtained in immunodeficient mice following intrastriatal implantation of cell populations as indicated in the Methods. A lentiviral vector expressing GFP under a PGK promoter (gift from M. Sadelain) was used for cell labelling and sorting. Cytogenetic analyses were conducted using standard methods at the Cytogenetics Core facility at Sloan Kettering Cancer Center. Knockdown experiments were performed using lentiviral vectors expressing shRNA for *NOTCH1* or *VEGFR2* (Santa Cruz). All experiments were carried out in triplicates or greater. Data are expressed as mean $\pm$ s.d. *P* values were determined following two-tailed student's *t*-test. A *P* value of <0.05 was considered significant. Tissues were obtained after patients' written consent under a protocol approved by the institution's Institutional Review Board.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Information Microarray and CGH data are deposited in NCBI's Gene Expression Omnibus (GSE24244, GSE24446, GSE24452, GSE24557 and GSE24558). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to V.T. (tabarv@mskcc.org).

Methods

Tissue processing. Surgical specimens were collected from the surgical suite at Memorial Sloan Kettering Cancer Center, following diagnostic confirmation by a neuropathologist. Tissues were obtained after patients' written consent under a protocol approved by the institution's Institutional Review Board. Tumours were cut mechanically first (McIlwain Tissue Chopper) then dissociated into single cells with Liberase Blendzyme 1 (Roche) as described previously²⁷. Single cells were blocked with human FcR (1:20, Miltenyi Biotec) at 4 uC for 20 min before incubation with primary antibodies for 30 min. Cells from xenografts were further blocked with 2,4-G2 (1:100; Santa Cruz Biotechnology) before incubation with antibodies. Cells were incubated with primary antibodies, washed and reincubated with appropriate secondary antibodies and resuspended in FACS buffer²⁷ (containing 1× Ca²⁺/Mg²⁺-free HBSS (Invitrogen), 10 mM of HEPES, 0.156% of glucose and 0.5% of low endotoxin BSA fraction V, all from Sigma (Sigma-Aldrich), at a pH of 7.2) with 1 mg ml⁻¹ 7-aminoactinomycin D (7-AAD, BD Pharmingen) before analysis. Mouse IgG1 or secondary antibody alone served as control for unspecific binding. Samples were analysed on a FACS Aria flow cytometer with CellQuest software (BD Biosciences) and data were analysed using FlowJo software (Tree star). A minimum of 10,000 events were counted and cell surface expression was analysed in 7-AAD-negative live cells. Antibodies used include: phycoerythrinor allophycocyanin-conjugated anti-CD133 (1:20, Miltenyi Biotec); FITC-conjugated anti-CD144 (1:20, Abcam), anti-CD105 (1:20, BD Biosciences) and anti-CD31 (1:20, BD Biosciences); mouse anti-human CD31 (1: 40; BD Biosciences), mouse anti-human CD105 (1:40; Dako), mouse anti-human VEGFR2 (1:40; Abcam), mouse anti-human CD34 (1:20; Abcam), mouse anti-human CD144 (1:20; Abcam); mouse anti-human CD133 antibodies (AC141 and AC133 epitopes (1:20 each), Miltenyi). FITC-conjugated lectin and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated lectins were purchased from Vector and Sigma separately.

DNA and RNA preparation. FACS-sorted cell populations from 21 glioblastoma patients were used to extract total RNA using an Absolutely RNA Nanoprep kit (Stratagene) or an RNeasy Kit (Qiagen). All RNA samples were pre-treated with DNase. Sorted cell populations from eight glioblastoma patients were used to isolate genomic DNA using the Picopure DNA extraction kit (Molecular Devices), followed by phenol (Invitrogen) extraction.

In vivo studies. Adult female NOD/SCID or male NOD/SCID gamma (NSG) mouse (Jackson Laboratory) were anaesthetized with ketamine/xylazine (Hospira) and placed in a stereotaxic frame (Stoelting Company). Freshly sorted cells were injected into the right striatum immediately after sorting at the following coordinates (relative to bregma): AP = +0.5, ML = -2, and DV = -2.7. Animals received 10,000 cells each of CD133¹/CD144², CD133²/CD144¹ or DP. NSG mice received 500 cells each of CD133¹/CD144² or DP. DN cells were used in three separate doses (10,000, 50,000 and 100,000 cells). Animals were killed upon exhibiting symptoms. Some animals received FITC-conjugated lectin by retroorbital injection before killing. Total animals grafted *n* = 40.

The gel implantation assay was modified from ref. 11. Briefly, GBM- or DP- derived CD105¹ (10^6 or 2×10^6 per ml) were resuspended in Collagen IV (Chemicon). GBM- or DP-derived cell-gel suspension (500 μ l) was injected sub-cutaneously below the xiphoid in four or three mice separately. Some animals received TRITC-conjugated lectin by tail vein injection before killing. After transplantation (21 days) the implants were retrieved, fixed overnight in 4% (v/v) buffered formalin at 4 °C, embedded in Optimal Cutting Temperature Compound (O.C.T. compound, Sakura Finetek) and sectioned on a freezing cryostat (Leica) for histological examination. Animals were housed and cared for in accordance with the National Institutes of Health (NIH) guidelines for animal welfare and all animal experiments were performed in accordance with protocols approved by our Institutional Animal Care and Use Committee (IACUC).

Animal imaging. *In vivo* magnetic resonance imaging was performed on a Bruker Biospec 4.7-Tesla 40-cm horizontal bore magnet. The system is equipped with a 200 mT m⁻¹ gradient system. Examinations were conducted using a 72-mm birdcage resonator for excitation, and detection was achieved using a 3 cm surface coil. T2-weighted spin echo images were acquired consecutively using a rapid-acquisition relaxation enhanced sequence (RARE). Animals were anesthetized with 2% isoflurane in N₂/O₂ mixture.

Immunofluorescence. Primary antibodies were chicken anti-GFP (1:1,000; Chemicon), mouse anti-human CD31 (1:400; Abcam); mouse anti-human CD34 (1:400; Abcam), mouse anti-human CD105 (1:400; Dako); mouse anti-human vWF (1:100; Dako); mouse anti-human VEGFR2 (1:200; Abcam); mouse anti-human Ki67 (1:400; Dako); mouse anti-human NCAM (1:150; Santa cruz Biotechnology), mouse anti-human mitochondria (1:200; Chemicon), mouse anti-human nestin (1:400; Millipore), mouse anti-human nuclear antigen (1:500; Chemicon), rabbit anti-human GFAP (1:1,000; Chemicon), mouse anti-human Tuj1 (1:500; Covance), mouse anti-O4 (1:200; Chemicon), rabbit anti-human caldesmon (1:400; Novus Biology). The following secondary antibodies were used: Alexa Fluor 488-conjugated goat anti-chicken or mouse or rabbit (1:1,000), Alexa Fluor 555-conjugated goat anti-mouse or rabbit (1:1,000), Alexa Fluor 555-conjugated goat anti-mouse IgM (1:500), all from Molecular Probes (Invitrogen).

Cell culture and clonal assays. GFP labelling was obtained by incubation with a PGK-GFP lentiviral vector (gift from M. Sadelain). For sphere cultures, freshly sorted CD133⁺/CD144⁻, DP and CD133⁺/CD144⁺ cells were cultured under clonal conditions (1,000 cells per cm² or 5 cells per μ l) in low-adherence plates (Corning) and maintained in serum free-Neurobasal medium supplemented with N2 (Invitrogen), 2 mM L-glutamine, 20 ng ml⁻¹ recombinant human epidermal growth factor, and 10 ng ml⁻¹ recombinant human fibroblast growth factor 2 (all from Invitrogen). Neurospheres were reseeded every 5 days after dissociation with Accutase (Innovative Cell Technologies). For neural differentiation, CD133⁺/CD144⁺ cells were cultured in laminin coated plates (BD Biosciences) using NeuroCult NS-A Differentiation Kit (human) (Stem Cell Technologies).

For endothelial progenitor cells, freshly sorted DP or CD144⁺/CD133⁺ cells were seeded on human fibronectin-coated plates (BD Biosciences) at a density of 10^5 ml⁻¹ with endo-cult liquid medium Kit (Stem Cell Technologies) for propagation. DP, CD144⁺/CD133⁺ or CD133⁺/

CD144²-derived DP cells were grown to 75% confluence and switched to M199 medium (Invitrogen) for quantification of endothelial differentiation as described previously¹⁹. GBM-derived CD105¹ cells were grown in M199 medium¹⁹ for 2 days before FACS analysis. The functional assay for endothelial cells was performed by incubation of cells with 10 mg ml⁻¹ of DiI-labelled acetylated low density lipoproteins (DiI-AcLDL) (Molecular Probes, Invitrogen) for 4 h.

For DP induction culture, GFP-labelled CD133¹/CD144² were co-cultured with tumour cells at a 20:1 ratio in N2 medium. The CD133¹/CD144²-derived DP cells were sorted by FACS after 5 days for further characterization. A minimum of 100 cells were counted in triplicate assays. They were cultured in three-dimensional collagen gel²⁰. For differentiation of CD133¹/CD144² to endothelial cells, tumour endothelial cells and GFP labelled CD133¹/CD144² cells or control cells were resuspended in endo-cult medium and grown on fibronectin coated plates for 7 days at a ratio of 100:1. Single cell clonal assays were performed by seeding freshly sorted single GFP-labelled cells on multi-well plates. Wells containing single green cells were identified and monitored until clone formation is established. Single-cell-derived clones were further sub-cloned and propagated twice, dissociated and seeded under neural and endothelial differentiation conditions as described above. Human umbilical cord-derived CD133¹ endothelial precursor cells (Biochain) or human dermal fibroblasts (Cell Applications) were maintained as per manufacturer instructions and used as control in clonal analysis.

Inhibitor studies. For drug treatment assays, cells were cultured in DP induction medium or endothelial differentiation medium containing 5 mM of the γ -secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester, Sigma-Aldrich) or 1 mg ml⁻¹ of bevacizumab (Genentech). Treated cells were analysed by FACS analysis after 48 h incubation. VEGF was measured in the culture medium with a human VEGF ELISA Kit (Invitrogen) following the manufacturer directions.

Knockdown studies. GBM-derived fresh DP and GFP-CD133sp cells were infected with shRNA virus targeting *VEGFR2* or *NOTCH1* or a control virus (all from Santa Cruz Biotechnology). NOTCH1 shRNA lentiviral vector mix contains three target-specific constructs: CACCAGTTTGAATGGTCAATTCAA GAGATTGACCATTCAAACTGGTGTTTTT; CCCATGGTACCAATCATGA TTCAAGAGATCATGATTGGTACCATGGGTTTTT; CCATGGTACCAATC ATGAATTCAAGAGATTCATGATTGGTACCATGGTTTTT. VEGFR2 shRNA lentiviral vector mix contains three target-specific constructs: ACTGTGGTGATT CCATGTCTTCAAGAGAGACATGGAATCACCACAGTTTTTTT; ACTTGTA ACCGAGACCTATTCAAGAGATAGGTCTCGGTTTACAAGTTTTTTT; CACC TGTTTGCAAGAACTTTTCAAGAGAAAGTTCTTGCAAACAGGTGTTTTT.

The infected cells were selected with 2–4 mg ml⁻¹ puromycin (Santa Cruz Biotechnology) and used for FACS analysis and/or collected for RT-PCR as described above after 5 days in selection.

In vitro angiogenesis assay. Intracellular vacuole formation was evaluated by culturing CD133¹/CD144²-derived DP cells in three-dimensional collagen gel as described in ref. 20. Tubular

network formation was assessed by culture in growth factor reduced Matrigel assay Kit (BD Biosciences) following the protocol from ref. 19.

Cytogenetic analyses and genomic PCR. Fluorescence *in situ* hybridization was performed using BAC clone RP11-339F13 and PAC clone RP5-1091E12 spanning the *EGFR* locus in 7p11, both labelled with Red-dUTP, together with a chromosome 7 centromere repeat DNA probe labelled with Green-dUTP targeted at the centromeric region of chromosome 7 (7p11.1–7q11.1 D7Z1 alpha satellite region). FISH was performed on sorted cells post cytopsin on glass slides. A minimum of 100 cells in interphase were analysed. Human dermal fibroblasts (HDF) served as normal control. The false positive rates for FISH probes was determined as 1% (s.d. 5 1.3) and the cut-off level for the diagnosis of amplification was set at 5% (.3s.d.) ($n = 3$, total counted 2,000 control cells).

FISH on tumour sections, as reported in Supplementary Tables 1b and 2, was performed independently by the Clinical Cytogenetics Facility at Memorial Sloan Kettering Cancer Center, as part of a now routine molecular diagnostic test. The probe used is the 7p12 LSI *EGFR* and the 7p11.1–7q11.1 CEP (D7Z1 alpha satellite) dual colour probe, purchased from Abbott Molecular.

Fluorescence immunophenotyping and interphase cytogenetics, a technique combining immunohistochemistry for CD105 and FISH for *EGFR*, was carried out on 10-mm thick tissue sections. Normal human brain cerebral-cortex sections (Biochain) were used as controls.

In a copy number quantification reaction by real-time PCR, *EGFR* primers were designed based on published data^{6,28,29,30}. Genomic DNA (10 ng) from sorted cells or normal human brain was used as template to examine the copy number of exons 4, 9, 11 in the *EGFR* gene; *GAPDH* was used as reference gene. Each replicate was normalized to *GAPDH* to obtain a DC_i , and then an average DC_i value for each sample (from the three replicates) was calculated. All samples were then normalized to the calibrator sample (normal human brain) to determine DDC_i . Relative quantity (RQ) is 2^{DDC_i} , and copy number is $2 \times 3 \times RQ$. The *EGFR* copy number in each population was defined by the average of copy number from three exons. Error bars indicate the range of the data from the three exons in each of the three samples.

Karyotype analysis was performed on metaphase spread of FACS-purified cell subpopulations that were in culture for 3 days. The cultures were treated with Colcemid (0.1 mg ml⁻¹) for 1.5 h before *in situ* metaphase preparation according to standard cytogenetics procedures.

CGH studies. Comparative genomic hybridization (CGH) assay was performed by hybridizing genomic DNA from sorted cells with 44K human genome CGH arrays, and frozen banked whole tumour on 244K and 1M human genome CGH arrays (all commercial arrays from Agilent). DNA from sorted cells was prepared as described above. DNA extraction, purification, labelling and hybridization were performed at Sloan Kettering Cancer Center's Genomics Core Facility according to the manufacturer's instructions. Log₂ ratios were normalized by Lowess against probe intensity and mean %GC of the genomic region mapped to by the probe. Segmentation of normalized log₂ ratios was by Circular Binary Segmentation (CBS, R package DNACopy).

A separate method was used to investigate whether an amplicon identified by CBS in one cell fraction might be present in a minor subpopulation in other cell fraction at a level not detected by CBS. A region of interest (ROI) is defined by the boundaries of the amplicon detected by CBS. Then this region is investigated in the CGH profiles of the other cell fractions as follows: the $\log_2$ ratios of the N probes under the ROI (within amplicon boundaries) are compared to $\log_2$ ratios of all the other probes in the entire chromosome by Student's t -test (one-tailed). The observed t -score is then compared to the distribution of t -scores obtained by equivalently testing all other sets of N neighbouring probes in the chromosome. The ROI is considered to be significantly gained if the observed t -score is seen or exceeded in less than 0.1% of all other chromosomal regions.

Expression microarray studies of whole tumours. Gene expression profiling was performed for a subset of 16 tumours for which additional frozen material was available using exon expression arrays (Human Exon 1.0, Affymetrix). RNA was extracted, labelled and hybridized at Sloan Kettering Cancer Center's Genomics Core Facility according to the manufacturer's instructions. Data was normalized in a cohort of 80 gliomas using Aroma.affymetrix (R package aroma.affymetrix). Expression was derived for RefSeq transcripts, and multiple transcripts for the same gene were distilled to a single gene expression value by median.

Transcriptomal class assignment was based on the nearest centroid of the four transcriptomal classes reported in ref. 13, using the subset of 840 signature genes described by this study (Supplementary Table 6; http://tcga-data.nci.nih.gov/docs/publications/gbm_exp/). Distances to centroids were defined using Pearson correlation and class assignments made by the largest correlation value. If the largest correlation was ≥ 0.2 , the sample was labelled 'indeterminate'. Correlations and class assignments are given in Supplementary Table 6.

Microscopic analysis. Sections were examined with confocal laser scanning microscopy (Leica Microsystems; Carl Zeiss MicroImaging). The data was analysed with Velocity or LSM5 (Carl Zeiss MicroImaging) software.

Tumour microvessel density (MVD) was assessed by quantification of the numbers of CD31⁺ tumour vessels in pixels using MetaMorph (Molecular Devices) image analysis software using unbiased sampling.

Gene expression analysis and quantitative real-time PCR for sorted cell populations. Total RNA of four subpopulations from two specimens were hybridized with human U133-plus2 array at Sloan Kettering Cancer Center's Genomics Core Facility and according to the manufacturer's instructions. Reference databases, including one set of CD34⁺ human haematopoietic progenitor cells (GSM476781) and two independent sets of human embryonic stem cell-derived endothelial progenitors (GSM492830 and GSM492828) were downloaded from Gene Expression Omnibus database. The array data are analysed by Partek software.

The data from 11 samples were normalized by RMA algorithms and the tumour samples then assigned in four groups based on the expression of membrane markers CD133 and CD144. The gene list is created by ANOVA with unadjusted P value less than 0.05 and then used as input for unsupervised hierarchical clustering by using Euclidian similarity metric.

For RT-PCR, total RNA (100–300 ng) was reverse-transcribed using random- primer and superscript III (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed with an Applied Biosystems Prism 7900HT sequence Detection System using SYBR Green PCR Master Mix (Applied Biosystems).

Primers: *CD34* (F: TCTGATCTCCATGGCTTCCT; R: ACTGAGGCAACAG CTCAACC), *CD144* (F: TCGTCATGGACCGAGGTT; R: TCTACAATCCCTT GCAGTGTGA), *VEGFR2* (F: GCAGGGGACAGAGGGACTTG; R: GAGGCC ATCGCTGCACTCA), *CD31* (F: TTCCTGACAGTGTCTTGAGTGG; R: GCT AGGCGTGGTTCTCATCT), *CD133* (F: TCTGGGTCTACAAGGACTTTCC; R: GCCCGCCTGAGTCACTAC), *ACTIN* (F: GCCCGCCTGAGTCACTAC; R: GGAATCCTTCTGACCCATGC), *VEGFR1* (F: TCTCACATCGACAAACCA ATACA; R: GGTAGCAGTACAATTGAGGACAAGA), *VEGF* (F: CTACCTCC ACCATGCCAAGT; R: CCACTTCGTGATGATTCTGC).

Human angiogenesis PCR arrays (SABiosciences) were used to examine the expression profiles of angiogenic genes in sorted cell populations. Heat Map construction and analysis of qPCR data was conducted according to ref. 31.

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Chapter 5

5

Organotypic Explant Culture of Glioblastoma Multiforme and Subsequent Single-Cell Suspension

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Glioblastoma multiforme (GBM) is one of the most aggressive brain tumors. GBM cell lines used in laboratory studies are frequently passaged in various culture media at high proliferation rates, resulting in significant genetic and molecular alterations. Thus, data obtained in cell lines are often inapplicable to patient tumors. Furthermore, recent studies suggest that there is a stem cell–like hierarchy among GBM cell populations and a crucial role for tumor vasculature in stem cells, as well as tumor growth, which cannot be reproduced in cell line cultures. Our laboratory has developed a novel three-dimensional (3D) organotypic “explant” system of surgical GBM specimens that preserves tumor cells in their original milieu, as well as the cytoarchitecture of the tumor stroma. Our previous study on the role of Notch inhibition has demonstrated a definitive effect on the tumor endothelium that could only be highlighted by this system. In this unit, we describe a detailed protocol for preparing GBM explants, and discuss strengths, as well as limitations of the explant system as an in vitro 3D model of GBM. *Curr. Protoc. Stem Cell Biol.* 19:3.5.1-3.5.9. © 2011 by John Wiley & Sons, Inc.

Keywords: glioblastoma multiforme (GBM) organotypic cultures explants

Introduction

This unit describes a recently developed protocol for preparing organotypic cultures of glioblastoma multiforme (GBM) to explore basic glioma biology, as well as to utilize in preclinical screens for therapeutic agents. In this model, tumor tissue obtained directly from the operation room is dissected into small pieces or “explants” and are maintained in an air-liquid interface either on Millicell (Millipore) or Transwell (Corning) semiporous membranes (Figs. 3.5.1 and 3.5.2A). This model was originally designed for the study of normal physiological properties in central nervous tissue slices (Stoppini et al., 1991; Gähwiler et al., 1997). For the GBM explant culture, we use chemically defined media without additional growth factors or sera (Hovinga et al., 2010), to avoid selective growth of stem/progenitor cell population or their differentiation. The GBM explants are maintained for a relatively short period (up to three weeks) before they are harvested for downstream analyses including immunohistochemistry, flow cytometry, and neurosphere assay.

This unit begins with a method for establishing a GBM explant culture (Basic Protocol 1), followed by a protocol for dissociating the cells (Basic Protocol 2) to perform flow cytometry analysis and neurosphere assay.

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Figure 3.5.1 A diagrammatic representation of the organotypic explant culture system and the neurosphere (or “gliomasphere”) culture system of glioblastoma multiforme (GBM).

NOTE: The following procedures are performed in a Class II biological hazard flow hood or a laminar-flow hood.
NOTE: All solutions and equipment coming into contact with live tissue and cells must be sterile, and proper aseptic technique should be used accordingly.
NOTE: All incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

Figure 3.5.2 (A) Six explants from a GBM cultured on a Millicell insert placed on a 35-mm culture dish. (B) Heterogeneous tissues from a single GBM tumor on a 100-mm culture dish. Upper row from the left: gray normal-like tissue, orange normal-like tissue, creamy-white spongy (necrotic) tissue, and hemorrhagic tissue; lower row: soft and gelatinous tissue. (C-E) Hematoxylin and eosin staining of the gelatinous tissue (C), spongy tissue (D), and normal-like tissue (E). Scale bars: 100 μm . (F) ATP-based viability assay was performed on GBM explants cultured for 10 days in vitro. Data represents mean $\pm$ standard error ($n = 3$). (G) 3D reconstruction of confocal immunofluorescence images of CD31 (red) and nuclei (blue) of an explant (adapted from Hovinga et al., 2010). Grid: 50 μm .

Organotypic GBM explant culture

Laboratory studies of glioma biology have long relied upon the use of dissociated glioma cell lines, which are evaluated in isolation from the tumor microenvironment, and are extensively passaged in the presence of serum. In view of the growing awareness of the role of the tumor stroma (specifically, the vascular niche) to the integrity of the tumor stem cell pool (Joyce, 2005; Calabrese et al., 2007), we have optimized the organotypic explant culture model of GBM, in which the original cytoarchitecture of the tumor is maintained. The interface culture method (Fig. 3.5.2A) offers advantages in experiments that require sectioning of the tissue for morphological studies, or for biochemical studies that require larger amounts of tissue (Gähwiler et al., 1997). The opened interface of the culture also allows, at all stages of culturing, the tridimensional observation throughout the thickness of the explants, and has been used for transplantation studies investigating glioma cell migration in normal brain tissue (Jung et al., 2002). We have established correlations between the explants and their corresponding parent tumors, whereby histology, vascularity, and proliferation rates were shown to be similar (Hovinga et al., 2010). Explants can be used to analyze the impact of treatments, such as small molecule drugs and irradiation on tumor cells, as well as endothelial cells (Hovinga et al., 2010). Media can be used for ELISA or other studies to analyze cytokines and growth factors secreted by the tumor. Explants can also be dissociated into single-cell suspensions for flow cytometry analyses and neurosphere assays.

Materials

GBM (tumor) specimen on ice

Phosphate-buffered saline, calcium and magnesium-free (CMF-PBS; Invitrogen, cat. no. 21600)

Penicillin/streptomycin

Red blood cell (RBC) lysis buffer (eBioscience) Ice

Fibronectin (see recipe)

Explant culture medium (N2 medium; see recipe)

35- and 100-mm culture dishes Stereoscopic dissecting microscope

Leica KL 1500 LCD (150-watt cold light source) 27-G ½-in. needles

1-ml syringes

Millicell inserts (Millipore) Scalpel no.10 (BD)

Prepare the GBM explants

1. Place the tumor specimen in a 100-mm culture dish, and rinse the tumor with ice-cold CMF-PBS containing a high concentration of penicillin/streptomycin (3×).
The tumor specimen should be obtained as soon as possible from the operating room. If a waiting period is unavoidable, the specimen should be placed in a sterile container on ice.
2. Treat the tumor with RBC lysis buffer in the 100-mm culture dish to remove excess red blood cells, and then rinse twice with ice-cold CMF-PBS containing penicillin/streptomycin (3×).

3. Place the tumor under a dissecting microscope using a cold light source to illuminate the tumor. Carefully remove necrotic tissue and large vessels using two 27-G needles attached to 1-ml syringes (bend the needle with the bevel facing you). Transfer the viable tissue into a new 100-mm culture dish, and place the dish on ice. Add 3 ml of ice-cold CMF-PBS to avoid drying of the tissue.

A relatively small amount of tissue is required to prepare explant cultures (2 mm³ per explant). Tumors with extensive necrosis will yield less viable explants. With some experience, one learns to recognize areas of necrosis (friable avascular tissue) prior to explant preparation.

4. In a laminar flow hood, place Millicell inserts into 35-mm dishes and wet each membrane with 50 µl of 1 µg/ml fibronectin in CMF-PBS. Then add 1 ml of explant culture medium (without added growth factor or sera) underneath the membrane.
5. Under a cold light source, carefully cut the tumor tissue into small explants (2 mm³) using a pair of scalpels, and place up to six explants per membrane insert. Add 10 µl of medium over each explant to keep it moist but not “flooded” in solution.

Significant tissue heterogeneity is expected. Care must be taken to avoid bias when grouping the explants for each experimental condition (see Critical Parameters and Troubleshooting). We try to avoid bias by equally distributing explants originating from adjacent tumor regions into all treatment groups, and by preparing a sufficient number of explants (nine to fifteen explants) from randomly selected parts within the tumor for each treatment groups.

6. Refresh the medium and add 10 µl medium over each explant every other day. Change tips for each well to avoid possible cross-contamination.

Medium collected from the explant culture may be snap-frozen by liquid nitrogen and stored at -80°C for analysis of secreted factors.

Preparing single-cell suspension from GBM explants

This protocol describes a method to prepare single-cell suspensions from the cultured GBM explants. The cell suspensions may be analyzed by flow cytometry or neurosphere assay to assess the presence of “stem-like” cells. Cancer cells with stem-like properties have been characterized in various ways including (1) neurosphere formation, (2) multilineage differentiation, (3) active proliferation, and (4) tumor formation in vivo. Glioma cells fulfilling these characteristics were initially identified as CD133 cells (Singh et al., 2004; Beier et al., 2007), and more recently as CD133 /SSEA-1 cells (Son et al., 2009). A marker-independent method to identify glioma-initiating cells by intrinsic autofluorescence property is also reported (Clement et al., 2010). Ability for glioma cells to form neurospheres (or “gliomaspheres”) has been correlated to the poor prognosis in patients (Pallini et al., 2008), and a detailed protocol for neurosphere assay is described elsewhere (Ferrari et al., 2010).

Materials

GBM explants cultured on Millicell inserts (see Basic Protocol 1)

Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (CMF-HBSS)

Liberase I DNase I Glucose Ice

Scalpel no.10 (BD) Forceps

1.5 ml microcentrifuge tubes Microcentrifuge

FACS tubes with cell-strainer cap

1. Finely mince the explants using a pair of scalpels. Cut out the membrane from the insert, and transfer the membrane along with the tissue using clean forceps into a 1.5-ml microcentrifuge tube containing 1 ml of CMF-HBSS. Mix by inverting the tube several times.
2. Shake off the tissue from the membrane, and remove the membrane from the tube using forceps.
3. Add 0.4 mg/ml Liberase and 1 $\mu\text{g}/\text{ml}$ DNase I, and incubate at 37°C until tissue breakdown is evident (approximately 15 to 30 min); mix the tube every 5 min. Occasionally pipet a few times to further promote dissociation of cells.

Breakdown is evident when the solution becomes murky (cells entering suspension).

Alternatively, Accumax (Innovative Cell Technologies) can be used. In this case, centrifuge the tissue and remove 0.5 ml of the dissociation buffer from the tube, and add 0.6 ml of Accumax.

4. Centrifuge 5 min at 1200 rpm, 4°C , in a microcentrifuge.
5. Resuspend the tissue in 0.5 ml CMF-HBSS, and pipet up and down 20 times.
6. Filter through the cap of an FACS tube with cell-strainer to remove debris.
7. Transfer the suspension into a microcentrifuge tube and centrifuge for 5 min at 1200 rpm, 4°C .
8. Wash the cells with 0.5 ml CMF-HBSS containing 0.9 M glucose to remove small debris. Centrifuge for 5 min at 1200 rpm, 4°C .
9. Resuspend the cells in 100 to 150 μl HBSS, place the tubes on ice, and proceed to flow cytometry or neurosphere assay.

Typically, $0.5\text{--}1 \times 10^6$ cells are recovered from five explants.

Reagents and solutions

For culture recipes and steps, use sterile tissue culture-grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see SUPPLIERS APPENDIX.

Explant culture medium (N2 medium)

F12/DMEM containing:

1.55 g/liter glucose

2 g/ liter sodium bicarbonate 25 $\mu\text{g}/\text{ml}$ insulin

100 µg/ml human apotransferrin
 20 nM progesterone
 100 µM putrescine
 30 nM sodium selenite
 Titrate pH to 7.2 and filtrate
 Add penicillin-streptomycin (1)
 Store at 4°C for no longer than two weeks

Fibronectin, 1 mg/ml

5 ml Dulbecco's phosphate-buffered saline (DPBS; Mediatech, cat. no. 21031 CM) 5 mg BSA
 Mix and filtrate
 Reconstitute 5 mg fibronectin in the solution above (5 ml of DPBS containing 0.1% BSA)
 Divide into 250-µl aliquots Store up to 6 months at -80°C
Dilute at 1:1000 in DPBS before use

Commentary

Background Information

New agents with promising effects in in vitro glioma models have repeatedly failed to be efficacious in patients. These in vitro models have long relied upon the use of glioma cell lines, which are repeatedly passaged and cultured in various media. Phenotypic characteristics and the genetic aberrations found within these cell lines often bear little resemblance to those found within the corresponding primary human tumor. For example, 100% of rapidly replicating glioma cell lines are undergoing active cell cycle at any given time, while the proliferation indices of GBM are generally <20% in situ (Li et al., 2008). This may explain why most drug screens have historically identified agents with cytotoxicity against rapidly dividing cells. Recently, the concept of tumor stem cells offered a new paradigm of tumor biology, and glioma stem cell cultures have been characterized as a more robust model than traditional cancer cell lines (Galli et al., 2004; Lee et al., 2006). In this model, freshly dissociated GBM tumor cells are cultured in a serum-free medium containing EGF and bFGF, both as nonadherent, multicellular neurospheres (or “gliomaspheres”), and as an adherent monolayer in polyornithine/laminincoated dishes. These glioma “stem cell” cultures may be closer to the phenotype and genotype of the parental tumor, and are perhaps more suitable for chemical and genetic screens especially when grown adherently (Pollard et al., 2009), though additional data with clinical correlations are required. Despite the demonstrated usefulness of these glioma stem cell cultures, the cells are still grown in isolation from the tumor microenvironment, including stroma and the vascular niche, which has been increasingly gaining attention for its role in supporting the tumor stem cell pool (Joyce, 2005; Calabrese et al., 2007). The tumor spheroid system was developed to provide an

experimental model of intermediate complexity between the classical two-dimensional culture systems and *in vivo* tumors (Sutherland, 1988). Spheroid cultures are submerged in the medium, and are usually grown from established tumor cell lines or, less frequently, from primary tumor specimens and may show limited resemblance to the original tumor due to its prolonged passage in serum-containing medium. It has been shown, however, that the genomic profile of GBM was well preserved in this culture system (De Witt Hamer et al., 2008), perhaps due to its moderate proliferation rate. Our approach to the study of GBM in the context of the tumor environment was predicated on the preservation of cytoarchitecture and cell population phenotypes, as well as a proliferation index comparable to that seen in the original tumor. To this end, we have optimized the organotypic explant culture model of GBM. Using this system, we have demonstrated that tumor endothelial cells are critical components of the glioblastoma response to Notch inhibition, and mediate its effects on tumor proliferation and self-renewal (Hovinga et al., 2010). The explant system provides a suitable system to model cell-to-stroma, as well as cell-to-cell communications within the tumor and their response to clinical interventions. Specific limitations of the explant system should, however, be considered when designing experiments. For example, although the vascular structure is preserved in the explants, nutrients, drugs, and immune cells are no longer carried through them and thus are no longer functional as blood vessels. Therefore, care must be taken when designing experiments involving the blockade of circulation in the blood vessels (i.e., anti-angiogenic therapy).

Critical Parameters and Troubleshooting

GBM is recognized for significant heterogeneity at the tissue (Fig. 3.5.2B), cell population, molecular, and genetic levels. Some areas are firm while others are soft or gelatinous, and yet others are cystic with local hemorrhage. In addition, neoplastic cells infiltrating areas conserving normal brain tissue are always present. It should be noted that each of these different tissue types might differ in proliferation, cytoarchitecture, and survival under the explant culture condition. From our experience, the soft and gelatinous tissues are often densely populated with neoplastic cells (Fig. 3.5.2C), in contrast to the firm and spongy tissues, which are almost devoid of cells (Fig. 3.5.2D). Tissue with high cellularity usually exhibit excellent tissue maintenance and viability under the explant culture condition. Importantly, the soft and gelatinous tissue should not be confused with the soft normal-like brain tissue (Fig. 3.5.2E), which often exhibit low viability in culture (Fig. 3.5.2F). In addition to the overt tissue heterogeneity, there are also well known regional differences in gene expression, and non-uniform representation of key gene mutations and genomic alterations within the same GBM specimen (Inda et al., 2010). Such heterogeneity could potentially become the source of significant interexplant variations during downstream analyses. Care must therefore be taken to avoid bias in tissue heterogeneity when comparing the control versus treated groups of explants. We try to avoid bias by equally distributing explants originating from adjacent tumor regions into different treatment groups, and by preparing a

sufficient number of explants (nine to fifteen explants per treatment group) from randomly selected regions of the tumor.

It is critical to prepare explants at an adequate size to avoid artificial cell death and necrosis due to insufficient penetration of nutrient factors and oxygen deprivation. Extensive studies in the spheroid culture system have established that for most types of human tumor cells, the thickness of sufficient nutrient penetration ranges from 100 to 220 μm (Sutherland, 1988). Although the size of the explants (2 mm^2) in this protocol largely exceeds such range, we usually see an excellent viability throughout the thickness of explant, which may be in part because most explants flatten out along the membrane over the course of culture (Gähwiler et al., 1997; Hovinga et al., 2010). As for penetration of drugs, we have not directly measured levels but have demonstrated downregulation of downstream effectors of the Notch pathway upon the addition of small molecule Notch inhibitors (Hovinga et al., 2010). We have also successfully used toxin-conjugated antibodies that selectively attach to and kill endothelial cells throughout the explant thickness (Hovinga et al., 2010). To ensure penetration of macromolecules, such as immunoglobulins, however, it may be necessary to make smaller explants (about half the usual size) and make small incisions to allow sufficient penetration. We have not experimented with other techniques requiring access to individual cells, and thus tissue penetration, such as direct viral infection of explant tissue or the use of small RNA molecules.

Anticipated Results

The explant culture system is very robust and, in experienced hands, the likelihood of successfully establishing explant cultures for a given tumor exceeds 90%. The explants survived well and were kept in culture for up to 3 weeks. The original cytoarchitecture was preserved with strong similarities between the parent tumor and its corresponding explant (Hovinga et al., 2010). Endothelial hyperplasia and vascular glomeruloid bodies (Fig. 3.5.2C), highly characteristic features of GBM, were also preserved in explants (Hovinga et al., 2010). Three-dimensional architecture was preserved as seen in the maintenance of a highly branched appearance of the capillary network within the explant (Fig. 3.5.2G). Tumor cells in the explant exhibit a high proliferation rate as demonstrated by BrdU incorporation and Ki-67 immunostaining (Hovinga et al., 2010). For a quantitative analysis, we compared the number of proliferating cells and the number of endothelial cells in matched sets of original tumors and corresponding explants and observed essentially identical values (Ki-67 of 18.5% and 16.7% and CD105 of 10.1% and 9.3%, respectively; Hovinga et al., 2010). Immunohistochemistry or FACS analyses for markers, such as GFAP, Nestin, CD133, and in vitro uptake of BrdU also shows minimal variability over the 5 to 14 days period tested (Hovinga et al., 2010). These data demonstrate minimal changes in tumor cell composition, marker expression, and cell proliferation in our in vitro culture system over time.

Time Considerations

Depending on the size of the tumor, 30 to 120 explants can be prepared within 1.5 to 3 hr. For preparation of single-cell suspensions from the explants, it should take 1.5 to 2 hr to process <10 culture membranes (with five explants per membrane), and 2 to 4 hr to process >10 inserts. Counting and plating the cell suspensions for the neurosphere assay will take another 1 to 1.5 hr, and another 2 to 3 hr to complete the FACS analysis.

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Chapter 6

Inhibition of Notch Signaling in Glioblastoma Targets Cancer Stem Cells via an Endothelial Cell Intermediate

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Key Words. Cancer stem cells, Organotypic cultures, Endothelial cells, CD133 cells, Radiation, Notch signaling pathway

Glioblastoma multiforme (GBM) is a highly heterogeneous malignant tumor. Recent data suggests the presence of a hierarchical organization within the GBM cell population that involves cancer cells with stem-like behavior, capable of repopulating the tumor and contributing to its resistance to therapy. Tumor stem cells are thought to reside within a vascular niche that provides structural and functional support. However, most GBM studies involve isolated tumor cells grown under various culture conditions. Here, we use a novel three-dimensional organotypic “explant” system of surgical GBM specimens that preserves cytoarchitecture and tumor stroma along with tumor cells. Notch inhibition in explants results in decreased proliferation and self-renewal of tumor cells but is also associated with a decrease in endothelial cells. When endothelial cells are selectively eliminated from the explants via a toxin conjugate, we also observed a decrease in self-renewal of tumor stem cells. These findings support a critical role for tumor endothelial cells in GBM stem cell maintenance, mediated at least in part by Notch signaling. The explant system further highlighted differences in the response to radiation between explants and isolated tumor neurospheres. Combination treatment with Notch blockade and radiation resulted in a substantial decrease in proliferation and in self-renewal in tumor explants while radiation alone was less effective. This data suggests that the Notch pathway plays a critical role in linking angiogenesis and cancer stem cell self-renewal and is thus a potential therapeutic target. Three-dimensional explant systems provide a novel approach for the study of tumor and microenvironment interactions. *STEM CELLS 2010;28: 1019–1029*

Disclosure of potential conflicts of interest is found at the end of this article.

Introduction

The prognosis for the malignant brain tumor glioblastoma multiforme (GBM) has remained poor for decades, with a median survival of about 1 year [1]. In combination with surgery, the most effective treatment for GBM is radiation, but its efficacy is limited due to significant radiation resistance. A small subpopulation of 'stem-like' cells is now thought to contribute to this radioresistant phenotype and lead to repopulation of the tumor after treatment [2–4]. Brain tumor stem cells share characteristics with normal neural stem cells, such as neurosphere formation, the capacity to differentiate into multiple lineages and possibly interaction with a complex stem cell niche that includes endothelial cells [5–7]. In fact normal neural stem cells have been proposed as the potential cells of origin of glioblastoma [8, 9].

Notch signaling is an evolutionarily well-conserved pathway that plays a key role in many aspects of development such as cell differentiation, proliferation, and apoptotic events, but the specific effects are highly context dependent [10, 11]. There are four human Notch receptors that consist of an extracellular peptide containing epidermal growth factor (EGF) receptor-like repeats and a transmembrane peptide. They display both overlapping and distinct tissue distributions as well as both redundant and distinct functions [12]. Notch 1 and Notch 2 are the most ubiquitously distributed whereas Notch 2 and Notch 4 are more specifically expressed in vascular smooth muscle and endothelial cells [13]. Ligand binding via the Jagged or Delta-like family of membrane proteins leads to cleavage of the receptor by members of the A Disintegrin And Metalloprotease (ADAM) and γ -secretase families of proteases. This results in the release of the Notch intracellular domain (NICD), which translocates to the nucleus where it interacts with members of the C-Promoter binding factor (CBF1), Suppressor of Hairless, Lag 1 (Longevity Assurance Gene) (CSL) family and recruits multiple coactivators and corepressors leading to activation of target genes as well as NICD degradation. Notch is thought to target the transcription of multiple genes, including members of the basic Hes (helix-loop-helix family) and Hey (hairy and enhancer of split related with XRPW motif) [14]. The Notch pathway plays a major role in maintenance of the stem cell state in the nervous system [15, 16] and in the regulation of angiogenesis in normal development as well as tumors including glioblastoma [17–19]. It also interacts closely with the vascular endothelial growth factor (VEGF) pathway in modulating tumor angiogenesis and is increasingly viewed as an important therapeutic target. However, the specific effect of Notch inhibition on tumor endothelial cells, and perhaps consequently cancer stem cells, is poorly defined.

Emerging data suggests that glioblastoma stem cells may reside in a protective vascular niche that could contribute to cell fate decisions and survival [20]. Interactions between cancer stem cells and the vascular niche are thought to confer a survival advantage following therapeutic insults as well as allowing maintenance of the stem cell population and thus the ability to repopulate the tumor. A greater understanding of the role of the stroma in modulating malignant progression in glioma and other tumors has led to attempts at re-calibration of therapeutic strategies to target both cell autonomous and environmental factors.

Laboratory studies of glioma biology have long relied on the use of dissociated glioma cell lines, which are evaluated in isolation from the tumor microenvironment. In view of the growing awareness of the role of the tumor stroma [21], specifically the vascular niche, to the integrity of the tumor stem cell pool, we optimized the organotypic or ‘explant’ culture model in which the original cytoarchitecture, cell connectivity, and stroma—including blood vessels—are preserved [22, 23]. Using this system, we demonstrate that tumor endothelial cells are key components of Notch signaling in glioblastoma and likely mediate its effects on proliferation and self-renewal.

Materials and Methods

Culture of Primary Tumors as Organotypic/Explant Cultures

GBM tumor specimens were collected from the surgical suite and placed on ice in sterile phosphate-buffered saline (PBS). The tumor tissue was treated with RBC lysis buffer (eBioscience, San Diego, CA, ebioscience.com) to remove red blood cells and washed twice with ice-cold PBS. Using sterile 1 ml 27.5G syringes (BD Biosciences, San Jose, CA, <http://www.bdbiosciences.com>), the tumor tissue was dissected under the microscope into $\sim 1 \text{ mm}^3$ pieces (explants). Five explants were placed on a sterilized culture plate insert (Millipore, Billerica, MA, <http://www.millipore.com>) that was pretreated with $1 \mu\text{g/ml}$ fibronectin (BD Biosciences,) and placed in a 3-cm well. Culture media consisted of F12-DMEM (Gibco, Grand Island, NY, <http://www.invitrogen.com>) and N2 supplements (glucose, glutamine, sodium bicarbonate, $25 \mu\text{g/ml}$ of insulin, $100 \mu\text{g/ml}$ of human apotransferrin, 20 nM progesterone, 100 IM putrescine, 30 nM sodium selenite [all components from Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>]) at a pH of 7.2 [24–26]. Sterile N2 media was added in the lower well and 10 l of media were added to the surface of each explant. The media was changed every other day. Bromodeoxyuridine (BrdU; Sigma-Aldrich, St Louis, MO, <http://www.sigmaaldrich.com>, 10 IM) was added to the media 8 hours before harvesting.

Tissue Processing

Tissue was fixed in 4% *p*-formaldehyde overnight at 4°C , then transferred to 30% sucrose at 4°C . Optimal cutting temperature compound (Sakura Finetek, Torrance, CA, <http://www.sakura-finetek.com>) was used for embedding and 7 or 200 lm (for 3D reconstruction confocal microscopy) sections were cut on a cryostat (Leica, Deerfield, IL, <http://www.leica-microsystems.com>).

Fluorescence Immunohistochemistry

The sections were fixed with methanol for 5 minutes and permeabilized with acetone for 1 minute at -20°C. They were washed in PBS and blocked with 10% normal goat serum (Invitrogen) in PBS and 0.3% Triton X-100 for 1 hour. Primary antibodies were incubated overnight at 4°C and appropriate secondary antibodies (Alexa conjugates, Invitrogen) for 1 hour at 25°C. Slides were washed in PBS, counterstained with ϵ -4',6-Diamidino-2-phenylindole (DAPI) (Invitrogen) and mounted in 70% glycerol or Prolong Gold anti-fade reagent (Invitrogen) for confocal and fluorescence microscopy. Antibodies included: Ki67 (1:100 Dako, Glostrup, Denmark, <http://www.dako.com>), CD105 (1:100, Dako), CD31 (1:100, BD Biosciences), NICD (NICD 1:000, Abcam, Cambridge, MA, Cambridge, U.K., <http://www.abcam.com>) and Hairy and Enhancer of Split 5 (HES5) (1:100, Chemicon, Billerica, MA, <http://www.chemicon.com>). BrdU and terminal deoxynucleotidyl transferase deoxyuridine triPhosphate (dUTP) nick end labeling expression was measured using the 5- bromo-2'-deoxy-uridine Labeling and Detection Kit I, and the POD (Horseradish peroxidase) In Situ Cell Death Detection Kit (both Roche Applied Science, Indianapolis, IN, <http://www.roche-applied-science.com>) according to manufacturer's guidelines.

Image Acquisition and Histological Analysis

Epifluorescence microscopy was performed using a BX51 microscope (Olympus, Center Valley, PA, <http://www.olympus-global.com>) equipped with a C4741-95 digital camera (Hamamatsu, Bridgewater, NJ, <http://www.hamamatsu.com/>). Images were processed using the SlideBook 4.2 image software (Olympus). Confocal microscopy was performed using a Leica TCS SP2 AOBS laser scanning DMRXA2 microscope. The maximum intensity and blend projections of the stacked images were computed using Bitplane Imaris 4.5 software (Zurich, Switzerland, www.bitplane.com). Thereafter, an isocontour model of blood vessels was generated to highlight the volume of the structures, then rotated, panned, and zoomed while recording the animation. The movie was later exported in .MOV format. Bright-field images were acquired using an Olympus IX71 microscope. Quantification of endothelial cells and ki67+ cells was performed on randomly selected high-power fields. On average, 4,000 cells per explant were counted.

Administration of Radiation and the γ -Secretase Inhibitor DAPT

The explants were allowed to attach to the insert overnight. A total of 25 μ g/ml of the γ -secretase inhibitor DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester, Sigma-Aldrich) were added and media changed every other day. Radiation was delivered via X-RAD 225C irradiator (Precision X-Ray, Inc., North Branford, CT, <http://www.pxinc.com/>). For the combination group, the explants were pretreated with 25 μ g/ml

DAPT for 5 days then irradiated with 10 Gy. DAPT was maintained in the media for the next 5 days.

Each experiment represents a minimum of three (range: 3–5) different tumors, each of which is represented in three wells. In average each condition thus consisted of a total of 9–15 explants per tumor, with a range of 3–5 tumors per experiment. Explants are pooled in groups of 3–5 per well.

Neurosphere Assay and Flow Cytometry

The explants were dissociated into smaller pieces and placed in Hanks' balanced salt solution (HBSS, Invitrogen) containing 1 mg/ml DNase I (Roche Applied Science) for 15–30 minutes at 37°C. The tissues were then triturated every 5 minutes and ultimately passed through a 35- μ m cell strainer (BD Falcon, www.bd.com). The cells were washed using sterile 0.9 M sucrose in HBSS (pH 7.3) and resuspended in N2 media containing 20 ng/ml basic fibroblast growth factor-2 and EGF (both from R&D Systems, Inc., Minneapolis, <http://www.rndsystems.com>). The cells were plated in ultralow attachment plates (Costar, Lowell, MA, www.colepalmer.com) at high density (20,000 cells per milliliter) for 3 days. Primary neurosphere formation was obtained readily but was often associated with nonviable clumps. After clearing agglutinating debris, we re-plated the cells at clonal density (100 cells per milliliter) and formed secondary neurospheres over 10–14 days.

For flow cytometry, the explants were dissociated in Liberase (400 μ g/ml, Roche Applied Science). Cells were blocked with 1:10 FcR blocking reagent (Miltenyi, Auburn, CA, <http://www.miltenyibiotec.com>) for 15 minutes at 4°C and labeled with CD133 antibodies (AC141 and AC133 epitopes (1:1), Miltenyi) for 30 minutes at 4°C. Flow cytometry analysis was performed using FACSCalibur flow cytometer (BD, Franklin, NJ ([bd.com](http://www.bd.com))) and dead cells were excluded using 7-amino-actinomycin D (BD Pharmingen, San Diego, CA, http://wwwbdbiosciences.com/index_us.shtml).

Clonogenic Assays

We established three different cell lines from fresh glioblastoma specimens [27, 28]. Briefly, surgical specimens were dissociated and cultured in N2 media under neurosphere conditions and different treatment conditions (DAPT, radiation and/or combination). Formed neurospheres were passaged for two to four times, and then dissociated and replated at clonal density (100 cells per milliliter). To further confirm clonogenicity under attached culture conditions, cells replated on culture were coated with polyornithine, laminin, and fibronectin. Colony formation was visualized with gentian violet staining.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted using Trizol (Invitrogen). A total of 0.5 µg of RNA was reverse-transcribed using SuperScript III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR) (Invitrogen). The resulting cDNA was subjected to real-time PCR amplification using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, <http://www.appliedbiosystems.com>). Primer sequences in supporting information Table 1.

Saporin-Conjugated Antibodies

The antibody CD105 (1:1,000) was incubated with goat antimouse IgG-saporin (ZAP, Advanced Targeting Systems, San Diego, CA, www.atsbio.com) in N2 media for 30 minutes at 37°C to allow binding and formation of a CD105 antibody-saporin complex [26], which was added to the explants or to hCMEC/D3 cell line (human cerebral microvessel endothelial cell line, courtesy of Babette Weksler) [29] used as control. Manufacturer directions were followed. As control, the CD105 antibody was incubated with a nontargeting goat IgG saporin that does not bind CD105. The antibody-saporin solution was injected into the explant under a dissecting microscope after gently incising the explant surface for better access.

VEGF Enzyme-Linked Immunosorbent Assay

VEGF in the culture media was measured as described previously [30], using a human VEGF enzyme-linked immunosorbent assay kit (Invitrogen).

Statistical Analysis

Statistical significance was determined using the student *t*-test (paired when indicated). *p*-values below .05 were regarded as significant. Errors are SEM. All experiments were performed in multiples ($n = 3-5$). Each *n* represents the number of independent tumors and a minimum of 10 explants per tumor.

Results

The Tumor Microenvironment Is Preserved in the Explant Model of GBM

Our approach to the study of putative cancer stem cells in GBM was predicated on the maintenance of the tumor stem cell niche and tumor stroma including endothelial cells in vitro. To this end, we optimized a system for the culture of organotypic brain slices, first described by

Stoppini for the study of the hippocampus [23]. This model was designed originally for the study of normal physiological properties in the central nervous system.

Tumor tissue was obtained directly from the surgical suite and was dissociated into small pieces or 'explants,' which were maintained in a transwell system, allowing their growth and maintenance in culture at an air-liquid interface. Chemically defined media was used without additional growth factors or sera. The explants survived well and were kept in culture for up to 3 weeks with good viability. They flattened out and grew slowly over the course of a week (Fig. 1A). The original cytoarchitecture was preserved with strong similarities between the parent tumor and its corresponding explant (Fig. 1B). This is best demonstrated in the preservation of tumor stroma, including a fibrillary GFAP+ background and tumor endothelium and pericytes (supporting information Fig. 1). Three-dimensional architecture was also preserved as seen in the maintenance of a highly branched appearance of the capillary network within the explant (3D-reconstructions in Fig. 1C and supporting information Movie). Endothelial hyperplasia and vascular glomeruloid bodies, highly characteristic features of GBM, were also preserved in explants (Fig. 1B, inset). Tumor cells in the explant exhibit a high proliferation rate as demonstrated by BrdU incorporation and Ki-67 immunostaining (Fig. 1D). For a quantitative perspective, we analyzed the number of proliferating cells and of endothelial cells in sets of original tumors and their corresponding explants and found essentially identical values (Ki-67 of 18.53% and 16.67% and CD105 of 10.13% and 9.29%, respectively). The process of explant culture is also highly efficient with the likelihood of successful explant derivation per tumor dissected exceeding 90% once protocols for tissue handling and culture were optimized. Occasionally explants containing significant areas of necrosis and pseudopalisades do not survive well and have to be discarded. Thus explants of glioblastoma maintain a significant similarity to the original tumor specimens.

We also analyzed variability within explants over the course of in vitro culture. Time course fluorescence-activated cell sorting analysis of four GBM samples on day1, day5, and day10 revealed no time-dependent changes of CD133+ cells in the explants ($n = 3$ sets of explants for each time point, Fig. 1E). Time course analysis of in vitro uptake of BrdU over time also shows low variability that remains below statistical significance ($p < .16$) (supporting information Fig. 2A). Immunohistochemistry for GFAP (Fig. 1F), Nestin, and CD133 (supporting information Fig. 2B) also shows minimal variability of expression over the course of 10–14 days.

This data suggests stability of tumor cell composition and marker expression as well as proliferation in our in vitro system over time.

Variability among tumors is a predictable element in this system, in view of the recognized genetic, vascular, and tumor cell heterogeneity in glioblastoma. A common occurrence is the variability of CD133 expression among different tumors (Fig. 1E) despite consistently rigorous gating. Exposure to radiation or chemotherapy treatments before surgery and specimen acquisition may result in significant selection and alterations in tumor response to in vitro. Stratification of tumors according to clinical and genetic variables is an area of intense research activity, which may lead to rapid testing and classification of GBM subtypes based on their transcriptome

Figure 1. The tumor microenvironment is preserved in the explant model of glioblastoma multiforme (GBM). (A): Bright-field image of GBM explants attached to a fibronectin-coated membrane. The explants flattened out and grew slowly over 7 days. (B): Hematoxylin and eosin staining of an explant at 7 days in vitro showing good preservation of cytoarchitecture and blood vessels, in comparison with its parent tumor. Inset shows a higher magnification of a glomeruloid body in both parent tumor and explant. (C): Immunohistochemistry for the tumor endothelial marker CD105 demonstrates maintenance of high vascularity. The three-dimensional structure of tumor vessels is demonstrated using a reconstruction of confocal immunofluorescence images of an explant in the right panel. CD31 labeling in red highlights the blood vessels, nuclei in blue (grid: 50 μ m). (D): Explants maintain a high level of proliferation, shown here at 5 days in vitro, by immunohistochemistry for BrdU and Ki67. (E): Flow cytometry analysis of CD133 in explants from four different tumors. The number of CD133⁺ cells within each tumor showed little variability over 10 days in vitro ($p > .05$ for all four tumors). Tumors often exhibited significant heterogeneity for CD133 expression. (F): Immunofluorescence images of GFAP show a well maintained cytoarchitecture and no significant change in GFAP expression in the explants over 10 days in vitro. Error bars are SEM [Scale bars: 500 μ m in (A) and (C); 100 μ m in (B) and (F); 50 μ m in (D)]. Abbreviations: BrdU, bromodeoxyuridine; DAPI, 4',6-diamidino-2-phenylindole; GFAP, Glial Fibrillary Acidic Protein.

Figure 2. Notch inhibition decreases the number and function of stem cells in glioblastoma multiforme (GBM) explants. (A, B): After 5 days of DAPT treatment, the explants were dissociated into single cells and grown under neurosphere conditions. A significant (84%) decrease in secondary neurosphere formation was observed [in (B), $p = .0018$]. (C, D): Flow cytometry analysis for CD133 in DAPT treated explants. Despite large variability in % CD133⁺ cells among the primary GBM tumor samples, there was a consistent relative decrease in the number of CD133⁺ cells averaging 50% on treatment with the Notch inhibitor and in comparison with controls. Scale bar in (A): 200 μ m. Error bars are SEM. Abbreviations: DAPT, N- [N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

and/or proteome profiles [31]. In this manuscript, a total of 23 tumor specimens were used, originating from nine male and 14 female patients, of whom nine had recurrent tumors and 14 were newly diagnosed. All tumors were WHO grade IV glioblastomas with one tumor known to have progressed from a previous WHO grade III anaplastic astrocytoma. All but one of the recurrent tumors had received irradiation and temozolomide at the time of tissue collection. Limited clinical cytogenetics data were available for 19 of 23 specimens (supporting information Table 2).

Notch Inhibition Decreases Self-Renewal in GBM Explants

Recent data suggests a role for Notch pathway in maintenance of self-renewal by CD133⁺ cells in vitro. We analyzed the impact of Notch inhibition on GBM by exposing the tissue explants to the γ -secretase inhibitor DAPT for 5 days. To analyze the impact of Notch inactivation on self-renewal, we performed secondary neurosphere formation assays at clonogenic density. Five days after continuous DAPT treatment, the explants were dissociated and placed under neurosphere conditions. A significant decrease in secondary neurosphere formation by an average of 84% was observed after DAPT treatment, compared with controls ($n = 3$, $p = .0018$) (Fig. 2A, 2B).

This was associated with a parallel decrease in the number of CD133+ cells as demonstrated by flow cytometry studies. Although the percentage of CD133+ cells varied among tumors, the relative decrease was very consistent (48– 53%) (Fig. 2C, 2D). These findings suggest that inhibiting Notch results in a decrease in self-renewal potential of tumor cells as well as a decrease in the number of CD133+ cells or downregulation of CD133 expression. The overall effect is compatible with a decrease in stem cell-like potential within the tumor explants.

The Effects of Notch Inhibition Is Mediated by the Loss of Endothelial Cells

The Notch pathway plays a key role in development and in the regulation of angiogenesis. The NICD and the downstream target gene, *Hes5*, follow a vascular and perivascular expression pattern in tumor explants as well as parent tumor tissue (supporting information Fig. 3A). Treatment of explants with DAPT for 5 days resulted in a loss of the CD105 expressing cells averaging 50% decrease but reaching up to 75% in some tumors (range: 1.1- to 4.1-fold decrease, $p = .0001$) as seen by immunohistochemistry and RT-PCR (Fig. 3A, 3B). Other endothelial

Figure 3. Notch inhibition exerts its effect in part by targeting endothelial cells. Following Notch inhibition with DAPT explants exhibit a decrease in expression of the endothelial marker CD105 as demonstrated by qRT-PCR [(A) 3.8-fold decrease, $p = .004$] and immunohistochemistry (B). (C): Downregulation of Notch target genes HES1 and HES5 ($p = .0071$ and $p = .00004$, respectively) on DAPT administration. (D): Immunohistochemistry for secondary neurospheres shows the absence of endothelial cells (CD105) and mesenchymal markers (CD73). Neurospheres are immunopositive for nestin and partially for CD133 (data not shown). Scale bars 100 μm in (A); 200 μm in (D). Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DMSO, dimethylsulfoxide; HES1, Hairy and enhancer of split 1; HES5, Hairy and enhancer of split 5; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

markers such as CD31, von Willebrand factor, and CD146 were also decreased (data not shown). This data suggest an antiangiogenic effect of Notch blockade with subsequent disruption of the vascular niche in the explants. The Notch downstream target genes HES1 and HES5 were significantly downregulated as expected (Fig. 3C). This effect was very consistent in all GBM's tested and reflects effective inhibition of Notch signaling. In view of the concomitant decrease in neurosphere formation as a consequence of Notch inhibition, we evaluated the secondary neurospheres for expression of endothelial or other stromal markers, specifically CD105 and CD73, and found them to be negative. Most neurosphere cells stained for nestin and CD133 (Fig. 3D).

Having shown above that Notch inhibition results in a decrease in neurosphere formation and CD133+ cells, we tested the hypothesis that it was in fact the effect on endothelial cells that mediates the decrease in brain tumor stem cell number and function.

We used a conjugated antibody system that targets CD105 (endoglin), a tumor endothelium marker that is widely expressed in glioblastoma [32, 33] and explants (Fig. 1C).

Anti-CD105 antibody is conjugated to a saporin toxin that is released in the cell on internalization of the antibody complex, resulting in cell death via ribosomal inhibition [34]. A brain endothelial cell line highly expressing CD105 (hCMEC, courtesy of Weksler) was used as a control and for dose optimization of the saporin-conjugated antibody. Significant cell death (37% compared with controls, $p = .028$) was obtained within 5 days of exposure to the anti-CD105 saporin complex (Fig. 4). Subsequently, explants were treated with anti- CD105-saporin for 5 days. Control explants received the CD105 antibody preincubated with a nontargeting goat IgG saporin complex. A selective and significant decrease in endothelial cells was observed in the

Figure 4. CD105-ZAP effectively kills human brain endothelial cells. The human brain endothelial cell line hCMEC expresses CD105 (red). Treatment with the anti-CD105 antibody conjugated to the saporin toxin but not the untargeted control resulted in cell death of the majority of human cerebral microvessel endothelial cell line (hCMEC) cells. The mitochondria activity based MTT assay confirmed this significant reduction in viability (44.9% reduction (611.6%), $n = 3$, $p = .029$, error bars represent SEM). Scale bars 25 μ m. Abbreviations: DAPI, 4',6-diamidino-2-phenyl- indole; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide; ZAP, saporin immunoconjugate.

treated explants ($p = .0024$) as shown by immunohistochemistry and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Fig. 5A). Other endothelial cell markers such as CD31 were also decreased. This was associated with a significant downregulation of the Notch effector Hes5 by 60% compared with untreated controls (Fig. 5B). Interestingly, the loss of endothelial cells resulted in a decrease in neurosphere-forming ability to <50% of controls ($p = .04$) (Fig. 5C). CD133 expression was analyzed by qRT-PCR and was found to be essentially unchanged (supporting information Fig. 3B). Flow cytometry post-ZAP treatment was very suboptimal possibly due to crossreaction between the saporin-conjugated anti-CD105 and the anti-CD133 antibodies, both being IgG1 isotypes. This was further associated with a decrease in secretion of VEGF into the media, similar in magnitude to that seen under conditions of pharmacological inhibition of Notch (Fig. 5D). Thus the loss of endothelial cells mimicked a Notch inhibition like state and reduced self-renewal suggesting that the mechanism underlying the effects of Notch blockade involves disruption of the vascular niche. The reduction in VEGF

Figure 5. Effect of selective elimination of endothelial cells in glioblastoma multiforme (GBM) explants. (A): Treatment with the CD105-saporin complex reduces endothelial cells as shown by immunohistochemistry and mRNA levels for CD105 (2.9-fold decrease, $p = .001$). (B): A decrease in HES5 transcript was observed as well after 5 days treatment with the CD105-saporin complex (right panel, 2.5-fold decrease, $p = .002$) suggesting suppression of Notch activity as a consequence of the loss of endothelial cells. (C): Disruption of the CD105⁺ endothelial cell population results in a significant decrease in neurosphere-forming ability and a tendency toward formation of smaller spheres. (D): Vascular endothelial growth factor (VEGF) secretion in the media was measured by enzyme-linked immunosorbent assay. Pharmacological suppression of Notch activity resulted in a decrease of VEGF secretion in the media of a similar magnitude to that seen after the CD105-saporin mediated loss of endothelial cells (30% decrease with and 43% decrease, respectively, $p = .038$). Scale bars: 100 μ m in (A); 50 μ m in (B); 200 μ m in (C). Error bars represent SEM. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; HES5, hairy and enhancer of split 5; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; ZAP, saporin immunoconjugate.

may be the result of the loss of endothelial cells although the possibility of an inhibitory effect by Notch on the VEGF pathway cannot be ruled out.

Notch Inhibition Enhances the Effect of Radiation in GBM Explants but not GBM Cultures

The impact of radiation on GBM is often studied in cell lines in vitro, and their radiation resistance is well documented, but the response of tumor cells within their native microenvironment is not well studied. This is particularly relevant in view of the data supporting a role for endothelial cells in supporting self-renewing cells in some brain tumors [6]. Explants from five different GBM specimens were pretreated with DAPT for 5 days followed by radiation with a single dose of 10 Gy. They were compared with explants from the same specimens receiving a single treatment (radiation or Notch) and untreated controls. The experiment was conducted over 10 days, to allow a 5-day pretreatment with the Notch inhibitor preceding radiation. Data analysis was performed 5 days postradiation. Single treatments, via DAPT or radiation, reduced proliferation in the explants by 50%. However, the combination of DAPT and radiation had a more profound effect, dramatically reducing the proliferation rate from 14.3% to 3.4% (Fig. 6A, 6B). Neurosphere formation was tested in all treatment and control groups (Fig. 6C, 6D). Notch inhibition resulted in a significant decrease in neurosphere-forming ability compared with controls ($p = .027$), whereas radiation alone did not have a statistically significant impact ($p = .450$). However, the combined treatment was most effective, reducing neurosphere formation from 28.57% in controls and 13.12% in DAPT alone to 8.12% ($p = .014$ and $.018$, respectively). The number of CD133+ cells decreased dramatically to 23.69% of control after DAPT and 33.42% after radiation ($p = .013$ and $.011$, respectively) (Fig. 6E). The combination treatment also resulted in a significant decrease compared with control (23.48% of the control value, $p = .041$). Although the combination group was more effective than radiation alone, its impact did not reach statistical significance ($p = .2$).

In comparison, we performed similar experiments on glioblastoma neurospheres dissociated from primary tumors and passaged for short periods (P2–P4). As noted previously, such neurospheres consist of nestin+ cells and are negative for endothelial or mesenchymal stroma cells. Neurosphere cultures were treated with DAPT, XRT or a combination, for the same duration of time as the explants, passaged and replated at clonogenic density. The impact on cell growth was analyzed by dissociating the spheres into single cells and analyzing surviving cell numbers. Our data shows a significant decrease in cell number in response to DAPT, and a more substantial decrease in the radiation and combination groups (supporting information Fig. 4A). Neurosphere formation was also dramatically reduced after radiation and the combination (from 18.8/1,000 cells to 1.9 and 1.5 neurosphere/1,000 cells plated in the radiation and combination groups, respectively, supporting information Fig. 4B). The number of CD133+ cells was highly variable among the tumors studied and trended down most significantly in response to the combination treatment (supporting information Fig. 4C). In comparison with the explant data, the impact of radiation

Figure 6. Notch inhibition enhances the effect of radiation in glioblastoma multiforme (GBM) explants. (A): Immunohistochemistry for Ki-67 (red) in explants exposed to DAPT, radiation or a combination treatment, demonstrating an antiproliferative response in all treatment conditions. (B): A quantitative assessment of the percentage of Ki-67⁺ demonstrates a decrease from $14.3 \pm 0.67\%$ to $7.2 \pm 1.19\%$ and $7.3 \pm 1.49\%$ following DAPT and radiation, respectively. The combination treatment reduced Ki-67- $3.4 \pm 0.98\%$. $p = .003$ (vs. DAPT), $p = .004$ (vs. radiation) and $p < 10^{-8}$ (vs. control). (C, D): Neurosphere-forming ability in control and treatment conditions demonstrates a lack of effect by radiation, while DAPT consistently reduced neurosphere formation. The combination treatment showed a more potent effect reducing neurosphere formation from 28.57% to 8.12% in controls. (E): Flow cytometry analysis of CD133 revealed a dramatic decrease in the combination treatments (23.5%, $p = .041$), but the effect was not significantly different compared with DAPT or radiation alone. Scale bars: 50 μm in (A); 200 μm in (C). Errors represent SEM. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester.

alone on cell number and self-renewal in the dissociated cell group is much more significant and is not enhanced by Notch inhibition. The increased resistance to radiation seen within the explants may be due to a variety of factors, including a protective role of the intact vascular niche on the tumor stem cells. It should be noted that unlike Notch inhibition, radiation treatment alone does not reduce the number of endothelial cells within explants (supporting information Fig. 3C,D). A more robust response to radiation is seen in the explants only in combination with loss of endothelial cells on Notch inhibition. In explants, there was a concomitant decrease in proliferation and the number of CD133+ cells in the group receiving radiation and yet an intact rate of neurosphere formation, suggesting that radiation alone does not impact putative cancer stem cell function. Alternatively, neurosphere-forming stemlike cells *in situ* may not always be CD133+, an observation with growing support in the literature.

Although the response to radiation is complex and warrants further investigation, this data supports a role for the microenvironment, specifically endothelial cells, in modulating the response to radiation and Notch inhibition. It further emphasizes the advantages of analyzing tumor response *in vitro* within the context of the tumor stroma.

Discussion

Studies using glioblastoma cell lines have long suffered from poor reproducibility in view of recognized alterations in genomic profiles and *in vitro* behavior. Work on cell lines or neurospheres highlights specific subpopulations of cells but fails to analyze the interactions among cell species within a tumor. This is particularly relevant in the case of the Notch pathway where cell-to-cell interactions play a key role in ligand availability and receptor activation. The effects of Notch signaling are highly context dependent and have thus to be analyzed for individual cell types in specific microenvironments. In addition, there is emerging data that suggests that tumor stem cells reside in a perivascular niche structurally and functionally. Here, we show that the organotypic explant model is a useful tool for studying GBM biology. The preservation of blood vessels and cell connectivity in their original context offer a definite advantage over the more commonly used cell lines or tumor sphere systems. The dual effect of Notch inhibition on the tumor stem cells and on endothelial cells as illustrated here could not be demonstrated in cell lines or xenografts, which may recruit host-derived endothelial cells. Thus our organotypic model is exquisitely suitable to study the biology of glioblastoma and to model therapeutic response *in vitro*. The description of stem-like cells in brain tumors and their potential contribution to therapeutic failure has led to significant expansion of research in this area [4]. The concept of cancer stem cells implies a hierarchical organization within the tumor, whereby tumor initiation and repopulation after cytotoxic therapy is ascribed to a small subpopulation of tumor cells. Although controversy remains about their lineage, phenotypic identification, and precise function [5], tumor stem cells are often defined *in vitro* as

CD133-expressing cells with neurosphere-forming ability. However there is growing data to support that neurosphere formation, self-renewal and tumor formation is not exclusive to CD133 expressing cells. The expression of this marker whose physiological function remains unknown may also be dynamic and is likely dependent on culture conditions. It has also been suggested that the similarities between cancer stem cells and normal stem cells could be due to tumor initiation in the normal stem cell compartment, rather than dedifferentiation of more mature cells [9, 35–37]. These data stress the importance of identifying pathways that specifically target the stem cell-like cell populations.

The Notch pathway is a particularly attractive candidate due to its key role in promoting self-renewal in normal neural stem cells [15] as well as its involvement in angiogenesis. In fact Notch signaling has been invoked as a possible mediator of refractoriness to antiangiogenic agents that target the VEGF pathway [38]. The impact of Notch activity in glioblastoma is not completely defined. Some data suggest a role in proliferation [39, 40] and several Notch components are frequently upregulated in GBM transcriptomes [41]. More recent data suggest that Notch inhibition depletes CD133+ cells in glioblastoma and promotes increased responsiveness to radiation [42, 43]. Data in medulloblastoma cell lines [43] also suggested that Notch inhibition results in depletion of stem-like cells and a decrease in tumor forming capacity. In our experiments, Notch inhibition in GBM explants resulted in a decrease in the number of putative cancer stem cells and in self-renewal, compatible with literature reports. However, it also led to a decrease in endothelial cells suggesting a possible relationship between an intact endothelial compartment and the stem cell or self-renewing population. In fact the selective elimination of endothelial cells from the explants resulted in a state highly similar to pharmacological Notch inhibition, with downregulation of Hes5 and a decrease in neurosphere-forming ability and CD133 cells. These data suggest that the suppression of cancer stem cell function and number is at least in part mediated by endothelial cells and that the effect of Notch inhibition may be in part secondary to its antiangiogenic effect. They also highlight the advantages of analyzing tumor response to pathway manipulation within its original microenvironment. Current literature supports the coexistence of tumor stem cells within a vascular niche [6] that could provide a survival and functional advantage for tumor repopulation. Endothelial cells also play a critical role in the maintenance of self-renewal and the neurogenic potential of normal neural stem cells [16, 44]. Further studies are required to further characterize the role of Notch signaling in modulating the interaction of endothelium and tumor stem cells.

Additional experiments also highlighted differences in response to radiation between the explants and dissociated tumor cells. There was a more significant response to radiation by the neurosphere cultures in comparison with the explants. Radiation alone had a modest impact on self-renewal capacity in the explants and did not lead to a significant change in endothelial cells. The addition of Notch blockade potentiated the explants' response to radiation but had a minimal effect on the dissociated cells. This again highlights the role of the microenvironment, specifically endothelial cells in modulating the response to radiation. It could also reflect the existence of heterogeneous GBM cell subpopulations in the explants, such as neurosphere-forming cells that are either noncycling under homeostatic conditions or capable of bypassing radiation damage

rapidly. This finding is compatible with clinical data whereby GBMs commonly exhibit a stable to a minor decrease in tumor volume followed by local recurrence after radiation. Resistant or previously non cycling stem-like cells may then re-enter the cell cycle and repopulate the tumor. An important caveat to this data interpretation is the possibility of selection of specific subpopulations under neurosphere culture conditions. In some of the experiments, there was a dissociated response between the number of CD133+ cells and neurosphere-forming ability. This may be in part due to the large variability of CD133 expression among tumors, but could also have implications as to the significance of CD133+ alone as a unique stem cell marker. Recent data suggest CD15 as a candidate marker for putative glioma stem cells [45], but that remains to be more extensively validated.

Notch inhibition can be achieved at various levels, including blocking ligand ubiquitination, altering receptor activation or more directly interfering with downstream events. Monoclonal antibodies that target Notch ligands such as DLL4 or that inactivate Notch receptors have also been described. Gamma-secretase is a member of the I-CLIP protease family that has numerous transmembrane targets other than Notch receptors and what has been referred to as “promiscuous cleavage specificity” [46]. In addition to Notch and its ligands, it can also target Erb-B4, E- and N-cadherins, CD44, the low-density lipoprotein receptor and Nectin-1 [47]. Most of the available gamma-secretase inhibitors (GSI), including DAPT, have no preference for substrates, as is commonly observed for small molecule inhibitors/drugs. However, there are ongoing efforts to modify known inhibitors and allow greater affinity to specific target substrates. These efforts are particularly active in the Alzheimer field where there are reports of compounds (such as NSAIDs [48] or coumarindimer based molecules [49]) that can selectively target Ab42 (amyloid b precursor protein) thus alleviating the potential long-term toxicity of global γ -secretase inhibition. Nonetheless several nonspecific GSI molecules are currently in clinical trials for different cancers. Alternate and potentially more specific means of blocking the Notch pathway are under development, such as monoclonal antibodies that target the Notch receptors or ligands and targeted tumor delivery.

From a therapeutic perspective, by targeting the stem cell and the endothelial compartments, Notch inhibition addresses two prime targets: tumor repopulation and angiogenesis. Our data suggest that the effect of radiation is clearly enhanced when carried out in a background of Notch inhibition. In the absence of Notch signaling, tumor stem cells lose their self-renewal ability and likely transit into a progenitor-like state that is more vulnerable to radiation. Future studies will address the *in vivo* role of combined radiation and Notch inhibition.

Conclusion

We have presented a novel adaptation of the organotypic culture system to the study of glioblastomas. The three-dimensional explant system preserves tumor cytoarchitecture and stroma including endothelial cells. This has allowed us to demonstrate that Notch inhibition also

results in a decrease in the number of endothelial cells within the tumor, which likely disrupts the perivascular niche harboring tumor stem cells. In fact selective elimination of endothelial cells via an antibodytoxin conjugate resulted in a Notch-inhibition like state and a decrease in self-renewal, suggesting that endothelial cells play an important role in tumor stem cell maintenance that is at least partially mediated by Notch signaling. The explant system further highlighted differences in the response to radiation between explants and isolated tumor neurospheres. Combination treatment with Notch blockade and radiation resulted in a substantial decrease in proliferation and in self-renewal in tumor explants whereas radiation alone was less effective. This data suggests that the Notch pathway plays a critical role in linking angiogenesis and cancer stem cell self-renewal and is thus a potential therapeutic target. Three-dimensional explant systems provide a novel approach for the study of tumor and microenvironment interactions.

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Disclosure of Potential Conflict of Interests

The authors indicate no potential conflicts of interest.

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Supplemental Figure 1. Immunohistochemistry sections of tumors and their corresponding explants demonstrate a fibrillary background and comparable GFAP and Nestin labeling. Scale bars are 50 μm in the upper panels and 25 μm in the lower panels.

Supplemental Figure 2. Assessment of time-dependent changes in explants cultured in vitro. A, Explants maintained a high level of proliferation rate shown by immunohistochemistry for BrdU (Error bars are SEM; ANOVA, $p=0.61$). B, Minimal variation was seen in the cytoarchitecture of the explants over 10 days in vitro as assessed by fluorescence immunohistochemistry for CD133 and nestin. Scale bars are 100 μm .

Supplemental Figure 3. A, The notch effectors NICD and HES5 are highly expressed around blood vessels in GBM. Fluorescence immunohistochemistry demonstrating the perivascular expression of NICD or Hes 5 (green) around CD105+ endothelial cells (red), in a GBM explant (DAPI nuclear stain in blue). Scale bars: left, 50 μm; right, 100 μm). B, CD133+ cells could only be analyzed by qRT-PCR which showed no significant changes after treatment with antiCD105 saporin for 5 days (n=3, p=0.73). C and D, Radiation alone had no effects on CD105+ endothelial cells as assessed by fluorescence immunohistochemistry for CD105 and qRT-PCR (n=3, p=0.50).

Supplemental Figure 4. The impact of Notch inhibition and radiation on isolated glioblastoma cells. A, All three treatments resulted in a significant decrease of cell growth (vs control), but DAPT did not significantly enhance the effect of radiation (radiation vs DAPT + radiation, $p=0.06$). B, Neurosphere formation was significantly decreased by radiation, but not by DAPT (vs control). Combination with DAPT did not significantly enhance the effect of radiation (radiation vs DAPT + radiation, $p=0.77$). C, CD133+ cells showed a decrease following each treatment with the combination treatment being most effective, but no significance was detected compared to the effect of radiation alone (radiation vs DAPT + radiation, $p=0.44$). D, Bright field images of clonogenic colonies visualized by gentian violet staining. Scale bar: 1mm.

Supplementary Table 1

β-actin	Fw	CACAG AGCCT CGCCT TTGCC
β-actin	Rv	CACAT GCCGG AGCCG TTGTC
HES5	Fw	CCCGG GGTTC TATGA TATTT G
HES5	Rv	GCCCT GAAGA AAGTC CTCTA CA
HES1	Fw	TTACG GCGGA CTCCA TGT
HES1	Rv	AGAGG TGGGT TGGGG AGT
CD105	Fw	GCGGT GGTCA ATATC CTGTC
CD105	Rv	GTTGAG GCAGT GCACC TTTT
CD31	Fw	TTCCT GACAG TGTCT TGAGT GG
CD31	Rv	GCTAG GCGTG GTTCT CATCT
CD133	Fw	TGACC CTCTG TGCTT GGTGC
CD133	Rv	TGGAA GCTGC CTCAG TTCAG GG

Supplemental Table 1. Primer sequences used in the manuscript.

Supplementary Table 2 Tumors used in the study

Tumor	Gender	Age	Stage	Radiation Therapy*	EGFR amplification	Other	Experiments
GBM1	M	69	new	no	n/a		Tumor microenvironment DAPT on explant CD105-ZAP on explant DAPT and XRT on explant XRT on explant
GBM2	F	35	recurrent	no	n/a	Progressed from AA	Tumor microenvironment DAPT and XRT on explant
GBM3	F	63	new	no	45%		Tumor microenvironment
GBM4	F	49	recurrent	yes	n/a	Progressed from AA	Tumor microenvironment DAPT on explant CD105-ZAP on explant Tumor microenvironment
GBM5	M	70	new	no	n/a		Tumor microenvironment DAPT on explant DAPT and XRT on cell line
GBM6	M	27	recurrent	yes	no		Explant time course
GBM7	F	52	new	no	100%		Explant time course
GBM8	F	32	recurrent	yes	no		Explant time course DAPT on explant
GBM9	F	82	new	no	no		DAPT on explant
GBM10	M	53	recurrent	yes	78%		DAPT on explant CD105-ZAP on explant DAPT and XRT on explant
GBM11	M	52	new	no	98%		DAPT on explant DAPT and XRT on explant
GBM12	F	69	new	no	n/a		DAPT on explant CD105-ZAP on explant
GBM13	M	62	new	no	no		DAPT on explant
GBM14	M	63	recurrent	yes	no	5 3 % amplification of CMS 7	DAPT on explant CD105-ZAP on explant DAPT and XRT on explant
GBM15	F	73	new	no	95%		DAPT on explant XRT on explant
GBM16	M	45	recurrent	yes	60%		DAPT on explant
GBM17	F	81	new	no	no		CD105-ZAP on explant DAPT and XRT on explant
GBM18	F	64	new	no	no	5 3 % amplification of CMS 7	CD105-ZAP on explant
GBM19	F	62	recurrent	yes	no	Trisomy CMS 7 in 12.5%	DAPT and XRT on explant Explant time course
GBM20	M	73	new	no	no	6 3 % amplification of CMS 7	DAPT and XRT on explant

Tumor	Gender	Age	Stage	Radiation Therapy*	EGFR amplification	Other	Experiments
GBM21	F	38	new	no	64%		DAPT and XRT on explant
GBM22	F	57	new	no	no		DAPT and XRT on explant DAPT and XRT on cell line
GBM23	F	47	recurrent	yes	92%		DAPT and XRT on cell line

(AA) Anaplastic Astrocytoma (WHO, grade III)
(*) Radiation therapy was administered to all patients concomitantly with Temozolomide

7

Chapter 7

General Discussion and Valorization

Angiogenesis inhibition in glioblastoma

Discussion

Due to its high vascularity the malignant brain tumor glioblastoma has been viewed as an ideal target for anti-angiogenesis therapy like bevacizumab. Randomized controlled trials to date unfortunately only showed a modest and unsustained impact on disease progression^{1,2}. Nonetheless bevacizumab continues to be used, especially in recurrent settings and in many clinician experiences some patients do seem to respond to the treatment next to the advantage of diminished need for steroids to reduce tumor-associated edema. The tumor micro-environment, that shelter the so-called brain tumor stem cells³, plays an important role in the resistance to anti-angiogenic treatment. The goal of this thesis was therefore further the understanding on the evasive mechanisms, particularly in the tumor micro-environment, that glioblastoma uses to overcome the resistance to anti-angiogenic treatment. By that I hoped to be able to identify patients that are more or less likely to respond to this treatment. An additional goal was to offer alternative ways of angiogenesis inhibition to make this an effective treatment for patients with glioblastoma. Which is the central hypothesis of this thesis.

VEGF secretion increases after radiation in glioblastoma cell lines.

Postoperative radiotherapy is standard treatment for patients with glioblastoma, however despite high dose radiation the tumor inevitably recurs. Due to high levels of secreted VEGF, glioblastoma is known for its extensive neo-angiogenesis. In the [first chapter](#) we investigated the VEGF secretion by glioblastoma cells with different radiosensitivity after irradiation. We showed that radiation induces a dose-dependent increase in VEGF- secretion in three glioblastoma cell lines: U87, U251 and U251-NG2⁴. Interestingly we did not find a decrease in cell number after irradiation, even after irradiation with as high as 20 Gy, which confirms high radioresistance. Since the cell line U87 was most resistant to irradiation and had the highest basal VEGF secretion it is tempting to suggest that enhanced VEGF secretion is involved in radioresistance. We concluded that irradiation induces significant enhancement of VEGF-secretion, which appears to be a general response in GBM cell lines we tested. Therefore we speculate that the radiation-enhanced VEGF-secretion decreases apoptosis and increases angiogenesis, both leading to GBM radioresistance. Angiogenesis inhibition can therefore be considered as an adjuvant for existing therapies for glioblastoma like radiotherapy.

Classical subtype and EGFR amplification are associated with a poor response to bevacizumab in the recurrent setting.

In [chapter two](#) we tried to identify patients that are likely to respond better or worse to anti-angiogenic therapy in glioblastoma, we analyzed the molecular profile of a cohort of patients at Memorial Sloan-Kettering center that received bevacizumab for recurrent glioblastoma. Our data suggested that patients with recurrent glioblastomas that exhibit EGFR amplification or

classical subtype per TCGA classification were associated with a shorter time to progression on bevacizumab, in comparison to tumors with a proneural, neural and mesenchymal subtype or tumors without EGFR amplification. Patients with a classical subtype had a median time to progression of only 2.8 months compared to 5.1 in the mesenchymal, 6.4 in the neural and 6.0 in the proneural subtypes. In patients with EGFR amplified tumor, the median time to progression was 3.7 months compared to 6.7 months in tumors that did not have EGFR amplification⁵. A worse outcome in EGFR- amplified and classical tumors was statistically significant on both univariate and multivariate analysis⁵. It is interesting to note that some patients with mesenchymal tumors seemed to do better on bevacizumab. In a few cases some patients managed to stay on this treatment for more than 18 months, suggesting that the mesenchymal subtype may be more suitable to this treatment. This is especially promising since this subtype was shown to have the worst prognosis and recurrent tumors have often shown a switch to this subtype⁶. These findings suggest that careful consideration should be given before starting recurrent glioblastoma patients on bevacizumab if the tumor is EGFR-amplified or exhibits a TCGA classical subtype. This is especially important since it has become evident in recent years that bevacizumab treatment is not without risk, in particular from wound breakdown and the possibility of inducing a more infiltrative aggressive tumor after treatment⁷.

Brain tumor stem cells can form endothelial cells

Although debatable, a small subset of cells in glioblastoma that express the cell surface glycoprotein CD133, that is also expressed in hematopoietic stem cells, seem to have some characteristics that qualifies them as brain tumor stem cells or BTSC⁸. In [chapter three](#) we show that in a number of glioblastoma samples the stem-cell-like CD133+ fraction includes a subset of vascular endothelial-cadherin (CD144)-expressing cells that show characteristics of endothelial progenitors capable of maturation into endothelial cells. Interestingly a subpopulation of endothelial cells within glioblastomas harbor the same somatic mutations identified within tumor cells, such as amplification of EGFR and chromosome 7 centromere. Extensive lineage analyses, including single cell clonal studies, further showed that a subpopulation of the CD133+ stem-like cell fraction is multipotent and capable of differentiation along tumor and endothelial lineages, possibly via an intermediate CD133+/CD144+ progenitor cell. We were able to demonstrate identical genomic profiles in the CD133+ tumor cells, their endothelial progenitor derivatives and mature endothelium further supporting the concept that endothelial cells can be formed by BTSC. When we exposed tumor samples to bevacizumab as well as by knockdown shRNA studies, we could demonstrate that blocking VEGF or silencing VEGFR2 inhibits the maturation of tumor endothelial progenitors into endothelium but not the differentiation of CD133+ cells into endothelial progenitors⁹. In clinical practice bevacizumab therapy is often interrupted by GBM progression characterized by a decrease in abnormal vascularity and significant invasive tumor behavior¹⁰. It is therefore conceivable that bevacizumab failure is due to the disruption of the dynamic relationships between these tumor fractions.

Brain tumor stem cells can be studied in an organotypic “explant” model

Emerging data suggests these brain tumor stem cells may reside in a protective vascular niche that this proximity to blood vessels could contribute to cell fate decisions and survival³. Interactions between cancer stem cells and the vascular niche are thought to confer a survival advantage following therapeutic insults as well as allowing maintenance of the stem cell population and thus the ability to repopulate the tumor. The commonly used preclinical models of glioblastoma cell lines are frequently passaged in various culture media at high proliferation rates, resulting in significant genetic and molecular alterations. This has led to the realization that data obtained in cell lines are often inapplicable to patient tumors. Furthermore, since the vascular niche seems to play a crucial role for BTSC and therefore tumor growth^{3,8}, this dynamic relationship can not be reproduced in cell line cultures. In [chapter four](#) we show a novel three-dimensional organotypic “explant” system of surgical GBM specimens that preserves tumor cells in their original milieu, as well as the cytoarchitecture of the tumor stroma¹¹. In this model, tumor tissue obtained directly from the operation room is dissected into small pieces called “explants” and are maintained in an air-liquid interface on semiporous membranes. This model was originally designed for the study of normal physiological properties in central nervous tissue slices¹². For the GBM explant culture, we use chemically defined media without additional growth factors or sera¹³, to avoid selective growth of stem/progenitor cell population or their differentiation that allows the study of BTSC. The GBM explants are maintained for a relatively short period (up to three weeks) before they are harvested for downstream analyses including immunohistochemistry, flow cytometry, and neurosphere assay. In our opinion this model is ideally equipped to study the BTSC niche and their pathways that can lead to therapeutic targets.

Notch inhibition inhibits brain tumor stem cells and angiogenesis in an organotypic “explant” model of glioblastoma

Notch signaling is an evolutionarily well-conserved pathway that plays a key role in many aspects of development such as cell differentiation, proliferation, and apoptotic events, but the specific effects are highly context dependent^{14,15}. Importantly the Notch pathway plays a major role in maintenance of the stem cell state in the nervous system^{16,17}, and in the regulation of angiogenesis in normal development as well as tumors including glioblastoma^{18–20}. In [chapter five](#) we showed that notch inhibition by the γ -secretase inhibitor DAPT resulted in decreased proliferation and self-renewal of tumor cells in our explant system but was also associated with a decrease in endothelial cells. Interestingly, when we selectively eliminated endothelial cells from the explants via a toxin conjugate, we also observed a decrease in self-renewal of tumor stem cells giving support to the “niche” concept. Further analysis revealed that γ -secretase inhibition but not bevacizumab repressed the transition between CD133+ BTSC to endothelial progenitor cells⁹. To mimic the clinical setting we tested the combination treatment with Notch blockade and radiation. The combination treatment resulted in a substantial decrease in proliferation and in

self-renewal in tumor explants while radiation alone was less effective ¹³. This data suggests that the Notch pathway plays a critical role in linking angiogenesis and cancer stem cell self-renewal and is thus a potential therapeutic target.

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Valorisatie

Gliomen zijn tumoren die in de hersenen ontstaan en zijn een relatief zeldzame vorm van kanker. In Nederland krijgen ongeveer 1100 volwassen patiënten deze diagnose en bij ongeveer 400 mensen gaat het om de meest kwaadaardige vorm glioblastoom. De prognose voor de kwaadaardige hersentumor glioblastoom blijft, ondanks de technologische en wetenschappelijke vooruitgang in de geneeskunde van de laatste 50 jaar, onveranderd slecht. De meeste patiënten overlijden na ongeveer 15 maanden en de impact die deze ziekte op patiënten en hun familie heeft is zeer groot. Veel van de huidige beschikbare chemotherapeutica hebben geen effect laten zien, ondanks dat in veel andere tumoren deze behandelingen wel effectief zijn gebleken. Dat komt waarschijnlijk doordat het lichaam de hersenen zeer goed beschermt tegen invloeden van buitenaf door de bloed-hersenbarrière wat in dit geval leidt tot minder goed doordringen van de behandeling in de hersenen.

Een van de eerste observaties van wetenschappers die tumoren onderzochten halverwege de vorige eeuw was dat deze een toegenomen vaatgroei hadden. De gedachte die onderzoekers dan ook snel kregen was om deze vaatgroei, die de voeding voor de tumor aanlevert, te remmen. De naam voor deze toegenomen vaatgroei naar tumoren is angiogenese en is een proces dat ook in de embryologie voorkomt als het menselijk lichaam gevormd wordt. Angiogenese remming werd al snel populair als behandeling tegen kanker en de eerste experimenten in muizen waren ook zeer succesvol. Een van de belangrijkste eiwitten in het proces van angiogenese bleek Vascular Endothelial Growth Factor (VEGF) te zijn en remmers van dit eiwit konden tumoren in muizen zeer effectief behandelen. De verwachtingen voor deze behandeling bij patiënten was dan ook hooggespannen en zeker bij patiënten met een glioblastoom omdat dit een zeer vaatrijke tumor is.

Sinds 2004 is de remmer van VEGF, die inmiddels de naam bevacizumab had gekregen, goedgekeurd voor de behandeling van verschillende vormen van kanker. De effectiviteit wisselde helaas sterk en bij de maligne hersentumor glioblastoom werd helaas geen toename in overleving gevonden bij grote gerandomiseerde studies. De patiënten hadden wel baat bij de behandeling omdat het langer duurde voordat de tumor progressie vertoonde en ook bleek dat door deze behandeling minder reactieve zwelling van het hersenweefsel optrad waardoor andere medicijnen om dit proces tegen te gaan minder nodig waren. Ook was de perceptie van veel neuro-oncologen dat sommige patiënten toch langdurig veel baat hadden van behandeling met bevacizumab. Deze observaties hebben ertoe geleid dat het een zeer gangbare behandeling voor patiënten met een glioblastoom in Amerika is, waarbij de tumor na de eerste behandelingen terugkomt.

De laatste jaren is er zeer veel onderzoek gedaan naar het gebrek aan effectiviteit van angiogenese remming en zo erachter te komen hoe deze middelen efficiënter gebruikt kunnen worden. De hoop hierbij is dat in de toekomst patiënten vooraf geselecteerd kunnen worden zodat ze

de hoogste kans hebben om te reageren om de beschikbare angiogenese remmers. Een van de belangrijkste ontdekking is dat het proces van angiogenese aanzienlijk ingewikkelder is dan aanvankelijk werd gedacht. Er zijn veel meer eiwitten bij het proces betrokken dan alleen VEGF en de omgeving van de tumor speelt een grote rol in de resistentie die vaak ontstaat zijn twee van de vele verklaringen die zijn gevonden. Het bleek ook dat de modellen die zijn gebruikt bij de eerdere succesvolle experimenten met VEGF remming geen goede correlatie hadden met tumoren bij patiënten. Desondanks blijft angiogenese een essentieel onderdeel bij het ontstaan van kanker. Als het lukt om hierin effectief te interveniëren zal dit zeker een gunstig effect hebben op de behandeling van de kanker en hersentumoren. De **centrale hypothese van de thesis** is derhalve: angiogenese remming in glioblastoom is een effectieve behandeling.

In **hoofdstuk een** wordt beschreven dat glioblastoom cellen na bestraling meer VEGF gaan uitscheiden. Dit kan duiden op een beschermmechanisme dat deze cellen gebruiken om te overleven en doet vermoeden dat bestaande behandelingen gecombineerd kunnen worden met een angiogenese remmer. In **hoofdstuk twee** wordt beschreven dat tumoren met een bepaald moleculair profiel een slechtere kans hebben om te reageren op de behandeling met een VEGF remmer. Dat is belangrijk voor patiënten omdat dit middel veel bijwerkingen heeft en daarom patiënten met deze specifieke tumoren beter geen VEGF remmer kunnen krijgen. Andere patiënten hebben dan waarschijnlijk meer kans om te reageren.

In **hoofdstuk drie** wordt getoond dat de het proces waardoor bloedvaten worden gevormd in tumoren ingewikkelder is dan aanvankelijk werd gedacht. Het blijkt dat tumor cellen zelfs soms eigenschappen hebben die lijken op stamcellen. Deze tumor stamcellen die vaak in de buurt van tumorvaten liggen kunnen dan bijdragen aan het vormen van bloedvaten en worden niet geremd door de meest gebruikte angiogenese remmers. In **hoofdstuk 4** wordt een model beschreven om tumor weefsel te onderzoeken waarin dit proces goed onderzocht kan worden omdat de relatie tussen bloedvaten en tumorcellen zo goed mogelijk in stand wordt gehouden. In **hoofdstuk 5** wordt vervolgens beschreven hoe een remmer van de tumor stamcellen (zogenaamde notch-remmer), de tumorstamcellen in het eerder beschreven model kan remmer en ook de bloedvatgroei remt. Hiermee is dus een potentieel nieuwe angiogenese remmer beschreven die beter zou kunnen werken bij patiënten in de kliniek.

Concluderend wordt in deze thesis zowel klinisch als preklinisch de het proces van angiogenese en de meest gangbare angiogenese remmer bij patiënten met de kwaadaardige hersentumor glioblastoom onderzocht. Daarnaast wordt een alternatieve manier van bloedvatvorming in glioblastoom geponeerd met een nieuw, meer klinisch relevant onderzoek model. Een nieuwe remmer voor angiogenese wordt beschreven die potentieel veel patiënten in de praktijk met deze verschrikkelijke tumor kan helpen. Deze onderzoeken verdiepen en vullen de bestaande kennis over angiogenese bij glioblastoom aan, en gaan hopelijk leiden tot verbetering van de behandeling van deze verschrikkelijke tumor.

Chapter 8

Summary en Nederlandse samenvatting

Summary

Taken together these findings highlight the complicated process that underlies angiogenesis in glioblastoma. The clinical challenges for the future will be to use this knowledge to overcome the therapeutic challenges to effectively use the angiogenesis inhibitors in the patients that will most likely respond to the treatment. Based on our data bevacizumab can inhibit the transformation from endothelial progenitor cells to endothelial cells, but has no influence the formation of endothelial progenitor cells from BTSC. It was possible to inhibit this step by a notch inhibitor that was found to be safe in a phase 0/1 trial²¹. It is therefore possible that notch inhibitors can augment bevacizumab to effectively inhibit angiogenesis in future trials. To test these hypotheses the organotypic “explant” system, that is ideally suited for these questions, can be used and may decrease the need for tests in animal models. The finding that EGFR amplification and classical subtype make glioblastoma less likely to respond to bevacizumab can be used to select patients on the basis of molecular characteristics of the tumor of the patient. Additional investigations are required to further validate this finding as well as to investigate the mechanistic basis for bevacizumab propensity to fail in the setting of amplified EGFR. One possibility is that EGFR amplified tumors have a higher percentage of endothelial cells that are derived from BTSC, but this remains to be proven. Despite the lack of effect of overall survival in trials to date, angiogenesis remains a hallmark of cancer and is considered a rate limiting step in carcinogenesis. To be able to effectively interfere in this process will have beneficial therapeutic effect, stand alone or in combination with cytotoxic treatments or immunotherapy, and is without a doubt worth the research effort.

Nederlandse samenvatting

Samengevat laten de bevindingen in deze thesis het gecompliceerde proces dat aan angiogenese ten grondslag ligt zien. De klinische uitdaging voor de toekomst is om deze kennis toe te passen om effectief gebruik te maken van angiogenese remmers bij patiënten die de meeste kans hebben er goed op te reageren. Onze data laat zien dat de VEGF remmer bevacizumab de transformatie van voorloper endotheelcellen naar endotheelcellen kan remmen, maar geen effect heeft op de vorming van voorloper endotheelcellen door hersentumor stamcellen. Dit was wel mogelijk met een notch remmer die veilig was bevonden in een fase 0/1 trial¹. Het is daarom mogelijk dat notch remmers gecombineerd met bevacizumab effectiever angiogenese kan remmen in toekomstige trials. Om deze, en andere hypothesen te testen is het in dit proefschrift beschreven organotypic “explant” model erg geschikt en kan ervoor zorgen dat minder dierproeven nodig zijn. De bevinding dat EGFR amplificatie en glioblastomen met het “classical” subtype minder goed reageren op behandeling met bevacizumab kan gebruikt worden om patiënten te selecteren voor behandelingen op basis van moleculaire eigenschappen van de tumor. Verder onderzoek is nodig om deze bevindingen te bevestigen en om een mechanistische verklaring te vinden voor het falen van angiogenese remming bij tumoren met EGFR amplificatie. Een mogelijke verklaring is dat EGFR geamplificeerde tumoren meer endotheelcellen hebben die van hersentumorstemcellen afkomstig zijn, maar dit is nog niet bewezen.

Ondanks het gebrek aan toename van overleving in trials met angiogenese remmers bij patiënten met een glioblastoom blijft dit proces een essentieel onderdeel bij het ontstaan van kanker. Als het lukt om effectief te interveniëren in dit proces, zal dat ongetwijfeld leiden tot een gunstig therapeutisch effect. Als alleenstaande behandeling of in combinatie met immunotherapie. Het is daarom zonder twijfel de wetenschappelijke inspanning waard.

Chapter 9

**Dankwoord,
Curriculum Vitae en
Publicatielijst**

Dankwoord

Dit proefschrift had er nooit kunnen komen zonder de hulp van zeer veel mensen, die ik allen zeer dankbaar ben. Desondanks wil ik er een paar uitlichten

First of all professor Tabar (chair department of neurosurgery MSKCC, New York). Dear Viviane, quite simply, I don't think I would have published any paper (and consequently no thesis) if it wasn't for your help. You have taught me to think academically at a higher level and to always be critical towards my own work. You have inspired me to keep trying to do better and come up with smarter solutions to the countless problems that we encountered in the laboratory and clinic in the years that we worked together in New York. I truly believe that you have made me a better researcher and clinician and I am forever grateful for that.

Next I would like to thank professor Gutin (retired chair of department of Neurosurgery, MSKCC in New York). I could not have wished for a better role-model than you. The seemingly effortless way, in which you inspire people and shaped the absolute world class neurosurgical department at MSKCC is almost unbelievable. You have inspired me to become the best neurosurgeon I can possibly be and whenever I face a difficult problem I wonder how you would have handled it. I benefit every day from the excellent surgical training you gave in New York and I could not have wished for a better tutor. You give meaning to the phrase "lead by example" and I can only hope to one day, as a neurosurgeon, to be able to walk in your footsteps.

Mijn promotor professor Temel. Beste Yasin, ik wil je hartelijk bedanken voor de manier waarop je me hebt opgevangen in Maastricht. Zonder jou, weet ik niet of ooit zou zijn gepromoveerd. Je aanstekelijke enthousiasme over onderzoek inspireert de gehele afdeling en gaat ongetwijfeld tot grote successen leiden.

Dr. W van Furth en professor. Dr. L Stalper. Beste Lukas en Wouter. Dankzij jullie ben ik bij dit interessante onderzoeksgebied betrokken geraakt en heb ik wetenschappelijke stages in San Francisco en New York kunnen doen. Over de jaren hebben jullie me altijd de juiste adviezen gegeven en gemotiveerd om verder te gaan met onderzoek. Ik heb veel geleerd van jullie verschillende aanpak en doorzettingsvermogen en heb goede herinneringen aan de jaren dat we samen onderzoek deden met dr. J Verhoeff en dr M Donker. Zonder jullie zou dit proefschrift er zeker niet zijn waarvoor mijn oprechte dank.

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The neurosurgical department at Memorial Sloan-Kettering cancer center. There are too many people I would like to thank here. Dr C. Brennan for his incredible teaching in tumor biology, dr Bilsky and dr Laufer for their leadership in spine surgery, all the lab workers, physician assistants, nurses, secretaries and scientific officers that I owe so much to. My sincere appreciation to you all.

Beste stafleden van de afdeling neurochirurgie van het MUMC+, dank voor het warme welkom in Maastricht. Ik heb het hier erg naar mijn zin een van de belangrijkste voorwaarde daarvoor vind ik leuke collega's. Ik zie ernaar uit om in de toekomst te blijven samen te werken.

Mijn opleider professor Vandertop. Zonder u was ik nooit neurochirurg geworden en had ik nooit het fellowship in New York kunnen doen. Mijn oprechte dank hiervoor.

De neurochirurgische staf van het voormalig Academisch Medisch Centrum en het VU medisch centrum. Hartelijk dank voor de rol die jullie in mijn opleiding hebben gespeeld. Opleiden vind ik een van de belangrijkste en moeilijkste taken van een academisch medisch specialist. Ik wil jullie bedanken voor de moeite die jullie erin hebben gestoken.

Mijn mede arts assistenten in het Academisch medisch centrum in Amsterdam. Ik wil jullie allen, en in het bijzonder dr. O van der Veer bedanken voor de samenwerking. Veel succes in de toekomst.

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Curriculum vitae

Koos Hovinga werd in Amstelveen geboren op 26 juni 1979. Hij groeide op in de Watergraafsmeer in Amsterdam waar hij na omzwervingen door heel Amsterdam, San Francisco en New York nu weer met zijn gezin woont. In 1991 startte hij op het Vossius gymnasium in Amsterdam waar hij in 1998 zijn diploma cum laude haalde. Na te zijn uitgeloot voor de studie geneeskunde studeerde hij in 1998 een jaar bewegingswetenschappen aan de VU in Amsterdam. In 1999 kon hij vanwege de cum laude regeling alsnog beginnen met de studie geneeskunde aan de Universiteit van Amsterdam. Tijdens zijn wetenschappelijke stage aan het einde van het 4^{de} jaar geneeskunde deed hij onderzoek naar de kwaadaardige hersentumor glioblastoom in laboratorium voor experimentele radiotherapie in het Academisch Medisch Centrum. Voor zijn scriptie hierover ontving hij de scriptieprijs van de universiteit van Amsterdam in 2005. Naar aanleiding van dit onderzoek deed hij nog een aanvullende wetenschappelijke stage in het brain tumor center in San Francisco onder supervisie van professor Haas-Kogan. Na zijn co-schappen in 2006 besloot hij aanvullende wetenschappelijk ervaring op te doen aan het Memorial Sloan-Kettering cancer center (MSKCC) in New York. Na een clinical rotation op de afdeling neurochirurgie verbleef hij twee jaar in het laboratorium van professor Tabar waar hij zich verder verdiepte in de moleculaire biologie van glioblastomen. Na een succesvolle periode met onder andere een publicatie in het wereldberoemde tijdschrift Nature kon hij in 2009 beginnen met zijn opleiding Neurochirurgie in Amsterdam. Na afronding van zijn opleiding in 2015 keerde hij terug naar MSKCC voor een fellowship neuro-oncologische neurochirurgie. In dit zeer prestigieuze fellowship leerde hij onder supervisie van professor Gutin tumoren in de hersenen en het ruggenmerg te opereren. In 2016 keerde hij terug naar Amsterdam waar hij als neurochirurg in het Slotervaart ziekenhuis en het Antoni van Leeuwenhoek ziekenhuis werkzaam was. Sinds 2019 is hij werkzaam in het Maastricht Universitair Medisch Centrum. Zijn primaire aandachtsgebieden zijn de schedelbasischirurgie en de oncologische neurochirurgie.

List of publications

EGFR amplification and classical subtype are associated with a poor response to bevacizumab in recurrent glioblastoma

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