TECHNISCHE UNIVERSITÄT MÜNCHEN

Institut für Allgemeine Pathologie und Pathologische Anatomie

(Direktor: Univ.-Prof. Dr. H. K. Höfler)

Fachgebiet für Neuropathologie

(Leiter: Univ.-Prof. Dr. J. Schlegel)

The Impact of Aldehyde Dehydrogenase-1 Expression on Glioblastoma Stem Cell Capacity

Michael Rasper

Vollständiger Abdruck der von der Fakultät für Medizin
der Technischen Universität München zur Erlangung des akademischen Grades eines
Doktors der Medizin
genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. E. J. Rummeny

Prüfer der Dissertation:

1. Univ.-Prof. Dr. J. Schlegel

2. Priv.-Doz. Dr. G. Weirich

3. Univ.-Prof. Dr. B. Hemmer

Die Dissertation wurde am 20.07.2011 bei der Technischen Universität München
eingereicht und durch die Fakultät für Medizin am 30.01.2013 angenommen.

Contents

1 Abbreviations

ALDH1	Aldehyde dehydrogenase-1
bFGF	basic fibroblast growth factor
CNS	Central nervous system
CO2	Carbon dioxide
Cy3	Cyanine3
DAB	3,3'-Diaminobenzidine
DAPI	4'-6-Diamidino-2-phenylindole
DEAB	4-diethylaminobenzaldehyde
DKFZ	Deutsches Krebsforschungszentrum
DMEM	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FCS	Fetal calf serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
GBM	Glioblastoma
HIF	Hypoxia inducible factor
HRP	Horseradish peroxidase
LOH	Loss of heterozygosity
MGMT	O6-methylguanine-DNA methyltransferase
mRNA	Messenger ribonucleic acid
MR	Magnetic resonance
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl fluoride
PTEN	Phosphatase and tensin homolog
RA	Retinoic acid
RAR	Retinoic acid receptor
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RXR	Retinoic X receptor

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SVZ	Subventricular zone
TBST	TRIS-buffered saline tween-20
TP53	Tumor protein p53
TRIS	Tris(hydroxymethyl)aminomethane
TSC	Tumor stem cell
WHO	World Health Organization

Units

C	Degree Celsius
g	Gram
kDa	Kilodalton
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
ng	Nanogram
U	Unit
μg	Microgram
μl	Microliter
μm	Micrometer
μM	Micromolar

2 Introduction

Next to diseases of the cardiovascular system, malignant tumors represent the second most common cause of death in Germany. In 2008, 216.010 people died of malignant neoplasms accounting for one out of four cases of all deaths (Statistisches Bundesamt, 2010). Tumors of the brain and the spinal cord account for a small proportion of all cancers (2%); approximately 8.000 people are diagnosed with such neoplasias per year in Germany. However, most of these tumors cause high mortality and significant patient morbidity. Brain tumors are the 7th-leading cause of overall cancer deaths in women (DKFZ Krebsatlas, 2010). In addition, neoplasias of the brain are the most frequent solid tumors in children (Deutsche Krebsgesellschaft, 2010) and the second-leading cause of cancer death behind leukemias in people younger than 40 years (Jemal et al., 2009).

Glioblastoma (GBM) is the most common and the most aggressive primary brain tumor in adults. Due to its infiltrative and diffuse growth pattern, the tendency to recur after surgical resection and a certain resistance to radiation and chemotherapy, GBM is associated with a poor prognosis despite intensive treatment. Recent research on tumor biology led to the notion that in certain tumors cells are hierarchically organized with so-called tumor stem cells resembling the behavior of non-neoplastic stem cells. These tumor stem cells are believed to be accountable for various clinical characteristics of malignant neoplasias including resistance to therapy and recurrent tumor growth after treatment.

In 2003, Singh et al. identified a tumor stem cell population in pediatric solid brain tumors (Singh et al., 2003). These cells could be described by their ability to grow to spheroids under certain culturing conditions and by the expression of specific proteins such as CD133 and nestin. Similar to the pediatric brain tumors examined by Singh et al., glioblastoma cells growing to spheroids and expressing the abovementioned stem cell markers have been observed. Other marker proteins for stem cell capacity in GBM have been described, including Sox-2 and Musashi-1. Nonetheless, the reliability of these TSC markers for the identification of tumor stem cells as well as the knowledge of their functional impact on stem cell char-

acteristics remains largely unclear; thus, the identification of new TSC markers in GBM seems necessary for an advanced understanding of tumor biology and for innovative therapeutic strategies.

The aim of the present investigation is to evaluate the relevance of aldehyde dehydrogenase-1 (ALDH1), a recently described and promising cytosolic TSC marker in a variety of other solid neoplasms, as a novel stem cell marker in human glioblastoma and to demonstrate the unique functional impact of ALDH1 expression on tumor stem cell properties.

2.1 Astrocytic brain tumors

Representing the most common malignancy of the central nervous system, glial tumors (gliomas) amount to 70% of all primary brain tumors in adults (Ohgaki and Kleihues, 2005). More than half of these tumors originate from astrocytic cells; these malignancies are refered to as astrocytic gliomas or astrocytomas. Regarding the typical growth pattern, invasiveness, tendency to recurr after therapy and prognosis, astrocytomas are categorized into four grades according to the World Health Organisation (Louis et al., 2007). Low-grade astrocytomas (WHO grade I and II) are declared as low malignant whereas high-grade astrocytomas (WHO grade III and IV) are defined as highly malignant. Pilocytic astrocytomas (WHO grade I) are relatively circumscribed lesions with little invasiveness and tendency to progress to more aggressive tumor forms; complete surgical resection is usually feasible leading to a cure. WHO grade II tumors are diffusely infiltrating low-grade astrocytomas which tend to progress to fatal high-grade (WHO grade III or IV) tumors. Due to their diffuse and infiltrative growth pattern, these tumors cannot be cured by complete surgical resection. Anaplastic astrocytomas (WHO grade III) and glioblastomas (WHO grade IV) are highly malignant brain tumors with a devastating prognosis and a challenge for oncologic treatment. Complete surgical resection is virtually impossible since these tumors diffusely invade the surrounding brain tissue. To date, the therapeutic regimen for high-grade astrocytomas consists of surgical gross resection followed by radiation and

chemotherapy. However, despite advances in surgical techniques, basic and clinical research within the last years, the prognosis of these brain tumors remains poor.

2.2 Glioblastoma

2.2.1 Epidemiology and etiology

Glioblastoma (GBM) commonly occurs in people aged from 45 to 75 years; the average age of onset is about 62 years (Counsell and Grant, 1998). In Europe and North America, the incidence of GBM is approximately 2-3 new cases per 100,000 people per year (Brown et al., 2009). The incidence of gliomas is 40 percent higher among men than women (Counsell and Grant, 1998). The underlying reasons for the observed sex differences remain largely unclear. The etiology of astrocytic brain tumors is not yet fully understood. For many years, efforts have been made to identify biologic or social factors contributing to gliomagenesis. Ionizing radiation has been found to be the only relevant environmental risk factor for brain tumors (Bondy et al., 2008). Therapeutic radiation in the childhood has been associated with an increased risk for meningeomas and gliomas (Neglia et al., 2006). Pesticides have long been suspected as risk factors for glial tumors; a 2004 study did not find any correlation between exposure to pesticides and glioma incidence (Ruder et al., 2004). Electromagnetic fields emerging from cellular telephones raised concerns over a possible influence on brain tumor risk. The INTERPHONE study group recently published data of more than 2700 glioma patients suggesting no correlation between cell phone usage and glioma risk (The INTERPHONE study group, 2010). Data on the influence of tobacco and alcohol use, head trauma, diet and medication on glioma development are rudimentary, further investigations are needed to clarify the influence of these factors on gliomagenesis.

2.2.2 Histopathological and molecular features

Glioblastoma is a diffusely infiltrating and highly malignant brain tumor accounting for approximately 65% of all astrocytomas (Ohgaki and Kleihues, 2005). Histopathologically, these tumors are defined by high mitotic activity, neovascularizations and broad necrotic areas within the tumor mass. Glioblastomas occur either *de novo* (primary GBMs) or by tumor progression from WHO grade II or III astrocytomas (secondary GBMs). Histopathologically, these forms can hardly be distinguished; more than 90% of all glioblastomas are believed to emerge *de novo* (Ohgaki and Kleihues, 2009). Primary and secondary glioblastomas partly differ in their genetic makeup. Loss of heterozygosity (LOH) of chromosome 10q occurs in both forms. TP53 mutations are common and early events in the progression of secondary glioblastomas. About 40% of all primary glioblastomas show amplification of the epidermal growth factor receptor (EGFR); this amplification rarely occurs in secondary glioblastomas (Schlegel et al., 2000).

The methylation status of the O6-methylguanine-DNA methyltransferase promoter (MGMT) is another important molecular marker in human GBM. A 2005 study showed that GBM patients with MGMT promoter hypermethylation had an improved overall survival under treatment with temozolomide (Hegi et al., 2005). This effect seems to be due to a MGMT mediated cellular resistance against alkylating agents (Pegg et al., 1983, Olsson and Lindahl, 1980). Promoter hypermethylation inactivates MGMT, a DNA repair gene, thus leading to enhanced chemosensitivity.

Recently, mutations of the cytosolic enzyme isocytrate dehydrogenase (IDH1) have been found to identify secondary glioblastomas evolving from low grade gliomas whereas primary GBMs were characterized by low frequencies of IDH1 mutations (Balss et al., 2008). Interestingly, WHO grade II and III tumors were found to largely carry IDH1 mutations (Gravendeel et al., 2010). Moreover, IDH1 mutations in WHO grade III astrocytomas and glioblastomas seem to be associated with better patient overall survival compared to IDH1 wild type tumors (Wick et al., 2009, Weller et al., 2009).

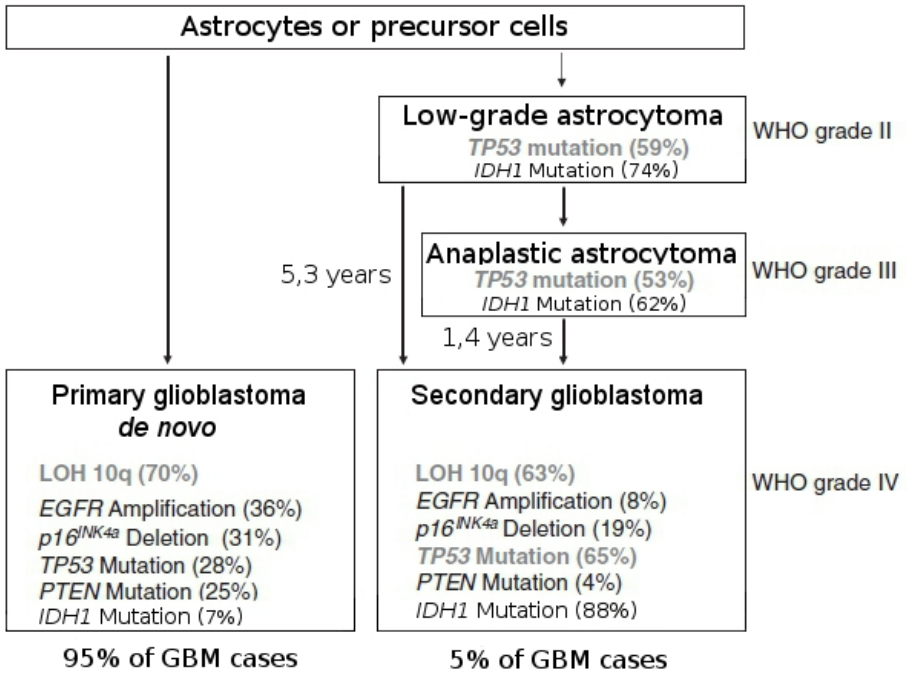

Figure 1: Model of glial tumor progression modified after Ohgaki et al. Typical timepoints and prevalences of genetic abberations during gliomagenesis (Ohgaki et al., 2005b).

2.2.3 Therapy

Prior to therapy, glioblastoma has to be diagnosed by stereotactic biopsy followed by histopathological workup. Currently the standard treatment of newly diagnosed glioblastoma consists of surgical gross resection, irradiation and chemotherapy. Surgical resection of glioblastoma is a challenging issue since this tumor grows diffusely and infiltrates eloquent brain areas. Preoperative MR-imaging helps to predict resectability; intraoperative imaging techniques such as intracranial ultrasound or fluorescence-guided surgery can help to improve resection results. Nevertheless, total resection of high-grade astrocytomas remains virtually impossible. Consequently, the surgical approach is only the initial step of glioblastoma treatment. Surgical therapy is usually followed by chemotherapy given concomitantly with and after radiotherapy (Stupp et al., 2005). Temozolomide, an orally given alkylating chemotherapeutic agent has largely displaced older chemother-

apeutics such as nitrosurea-based agents. Temozolomide was the first agent for which a level 1 evidence in the treatment of glioblastoma could be provided. A large multi-institutional phase III trial compared the treatment with temozolomide concomitant to radiotherapy with radiotherapy alone and found a significant advantage to the combination regiment (median survival time 14.6 vs. 12.1 months) (Stupp et al., 2005). Consequently, the standard treatment of newly diagnosed glioblastoma consists of concurrent temozolomide and radiotherapy followed by 6 cycles of temozolomide given for 5 days every 28 days. Innovative glioblastoma treatment strategies including targeted therapies or immunotherapies are under development and are still to prove their efficacy.

2.3 The tumor stem cell paradigm and glial tumors

Glioblastoma relapse occurs regularly after resection, irradiation, and chemotherapy. This could in part be due to the existence of so-called tumor stem cells (TSCs), a cellular subfraction within GBM contributing to recurrent tumor growth and resistance to drugs and irradiation. Within the last decade, TSCs have been identified and isolated in a variety of hematologic and solid neoplasms (Bonnet and Dick, 1997, Visvader and Lindeman, 2008). The characterization of these cells seems to be crucial for a better understanding of tumor biology and for the development of more efficient antitumor therapies. In the TSC paradigm, hematologic and solid tumors consist of heterogenous and hierarchically ordered cellular subdivisions. TSCs are believed to harbor the ability to keep a tumor alive and growing, possessing pluripotency, self-renewal, and resistance to chemotherapy and radiation therapy. This concept arose from the notion that a subpopulation of cancer cells shows similarity to normal stem cells (Reya et al., 2001). Leukemias were the first malignancies from which cells could be isolated that showed the potential to self-renew and to drive tumor formation and growth (Bonnet and Dick, 1997).

A stem cell subfraction has been described in brain tumors and especially in high-grade astrocytomas. Singh et al. were the first to identify and purify a popu-

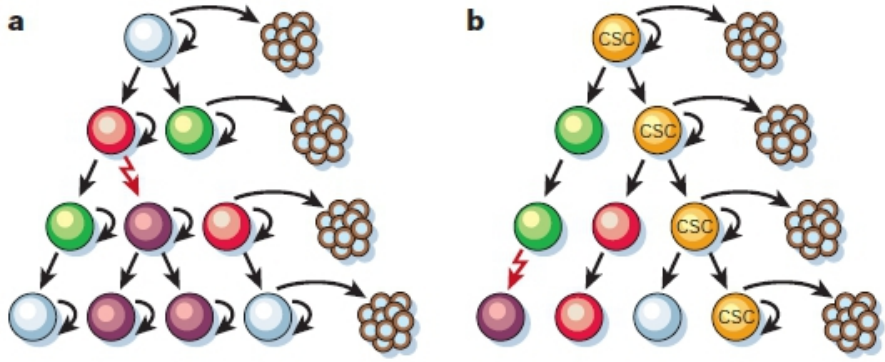

Figure 2: Two models of tumor heterogeneity after Reya et al., 2001. a) The tumor cells are heterogenous but all or many cells are proliferative and able to form new tumors. b) Tumor cells are heterogenous but only the tumor stem cell subpopulation (TSC) is proliferative and capable of growing to new tumors.

lation with stem cell properties in pediatric solid brain tumors (Singh et al., 2003). Those cells were identified by their ability to form spheroids (neurospheres) when grown under serum-free cell culture conditions and by the expression of CD133 and nestin. CD133 (also known as prominin-1), a 120-kDa pentaspan transmembrane glycoprotein, has been found to be a marker of stemness in hematopoietic (Yin et al., 1997) and neural cells (Uchida et al., 2000). Together with nestin, an intermediate filament protein expressed by undifferentiated neuroepithelial cells (Tohyama et al., 1992) and tumors of the CNS (Dahlstrand et al., 1992), CD133 has long remained the most important TSC marker in malignant glioma. To date, the functional role of CD133 in glioma stem cells remains largely unclear.

Other putative TSC markers in human brain tumors have been discussed. Sox-2 encodes a group of transcription factors that are activated at different times during cellular development (Yuan et al., 1995) and has been found in neural progenitor cells (Komitova and Eriksson, 2004). Ma et al. showed that Sox-2 mRNA expression increases with the grade of malignancy in WHO grades II, III, and IV gliomas (Ma et al., 2008). Musashi-1 encodes for various RNA-binding proteins that con-

tribute to the maintenance of an undifferentiated stem cell-like phenotype during CNS development (Okano et al., 2005).

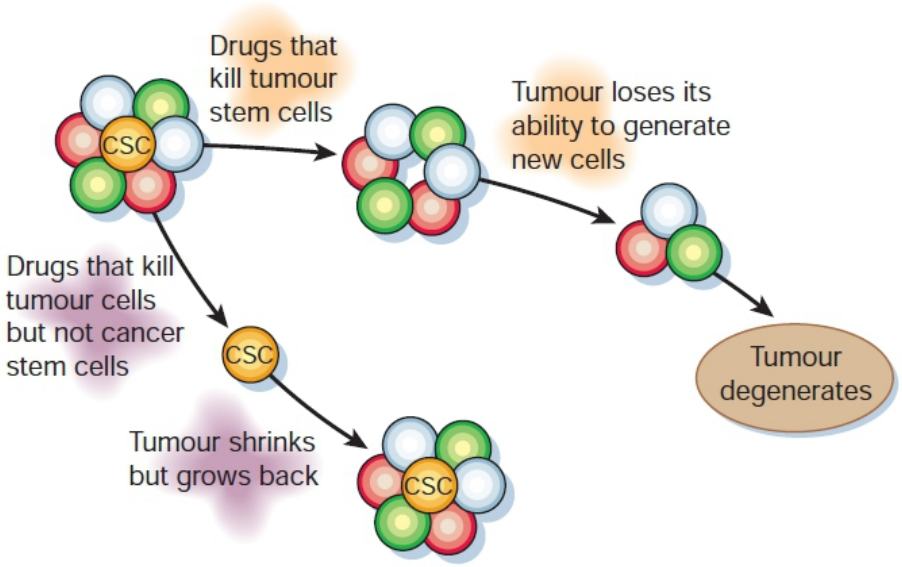

Figure 3: Model of cancer stem cell drug resistance after Reya et al., 2001. Tumor stem cells (TSCs) survive conventional therapies and consequently reconstitute the tumor mass.

Besides the expression of stem cell specific markers, the neurosphere formation assay is a well-established method to investigate functional properties of non-neoplastic neural stem cells and is also appropriate for the examination of brain tumor stem cells. This assay depends on the capacity of even single neural stem cells to grow to spheroids in the presence of mitogens such as the epidermal growth factor and fibroblast growth factor (Reynolds and Weiss, 1992). Accordingly, brain tumor stem cells can be identified by the ability to form tumor spheroids under similar culture conditions. Tumor stem cells show a low grade of cellular differentiation. Accordingly, the ability to differentiate into varying cellular sublineages under certain culturing conditions is a specific TSC feature (Wagers and Weissman, 2004, Suzuki et al., 2004, Woodbury et al., 2000). Brain tumor stem cells behave similar to neural stem or precursor cells including the potential

to mature into astrocytic, neuronal and oligodendrocytic cell types or their respective precursors (Corti et al., 2006). Consequently, glioblastoma TSCs can be identified by the low grade of cellular differentiation in combination with the ability to give rise to mature neural cell lineages.

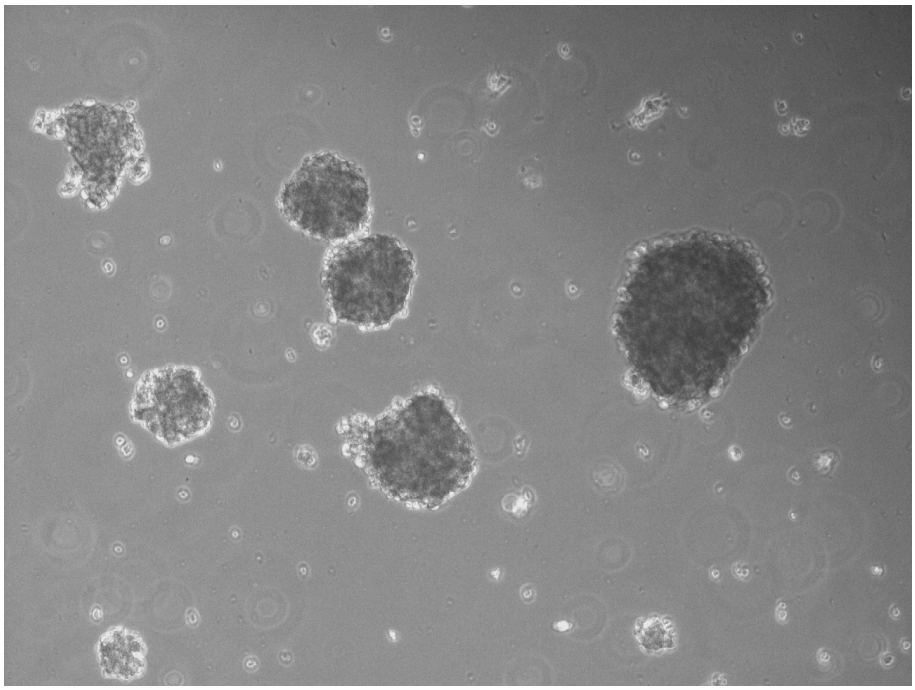

Figure 4: Tumor neurospheres formed by LN18 glioblastoma cells cultured in neurobasal medium supplemented with epidermal growth factor and fibroblast growth factor.

2.4 Aldehyde dehydrogenase-1 and tumor stem cells

Aldehyde dehydrogenase enzymes catalyze the NAD(P)-dependant oxidation of a variety of endogenous and exogenous aldehydes arising - beside others - from the oxidation of membrane lipids, amino acid and drug metabolism. In this context, aldehyde dehydrogenases play a critical role in the cellular defense against oxidative stress (Pappa et al., 2005). Aldehydes are highly reactive and long-lived agents; aldehyde toxicity is believed to be due to the formation of intracellular

adducts with a variety of cellular targets including nucleic and amino acids. These adducts presumably cause DNA damage, growth inhibition and cell death (Brooks and Theruvathu, 2005). To date, the human aldehyde dehydrogenase gene family consists of 19 genes (Marchitti et al., 2008); ALDH isoforms can occur in all subcellular regions and are expressed by the majority of human tissues including testis, brain, lens, liver, lung and retina. Class I aldehyde dehydrogenases (ALDH1) have been described as cytoplasmic stem cell markers in a variety of malignant tumors. As a member of the aldehyde dehydrogenase enzyme family, ALDH1 catalyzes the oxidation of intracellular aldehydes to their respective carboxylic acids, including the transformation of retinal to retinoic acid. Retinoic acid (RA) is a modulator of cell proliferation and differentiation that possibly contributes to the maintenance of an undifferentiated stem cell phenotype (Chambon, 1996, Tocci et al., 1996, Zile, 2001, Collins, 2002, Zechel, 2005). Jones et al. presented a method to isolate human cells via flow cytometry depending on the amount of cytosolic ALDH (Jones et al., 1995). They were able to enrich human hematopoietic precursor cells capable of reconstituting bone marrow in irradiated animals. Recently, Ginestier et al. found ALDH1 to be a stem cell marker in breast carcinomas with poor clinical outcome (Ginestier et al., 2007). Since then, ALDH1 has been described as a marker of stemness in other solid malignancies, including lung cancer (Jiang et al., 2009) and colorectal cancer (Huang et al., 2009). Due to their detoxifying activity, aldehyde dehydrogenases may play a role in cellular resistance to cancer therapeutics. ALDH1 was shown to facilitate resistance to alkylating agents such as cyclophosphamide (Magni et al., 1996) and oxazaphosphorine anticancer drugs such as ifosfamide (Sladek, 1999). Consequently, cancer stem cell chemoresistance might be mediated by high aldehyde dehydrogenase expression.

2.5 Aims

Being the most aggressive primary brain tumor, glioblastoma (GBM) is associated with high patient morbidity and poor prognosis. Regardless of intensive treatment

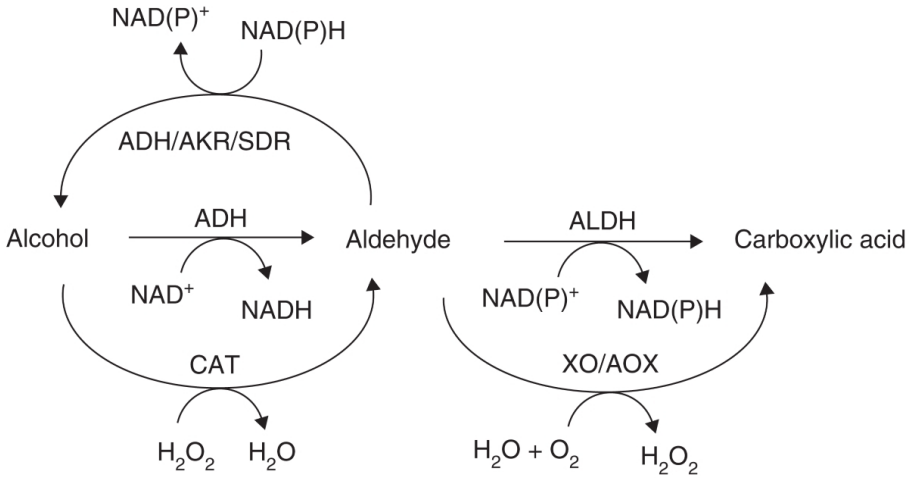

Figure 5: Aldehyde metabolism after Marchitti et al., 2008.

consisting of surgical resection, irradiation and chemotherapy, this brain malignancy regularly recurs within months after therapy. This observation might be due to the existence of a tumor stem cell subfraction within glioblastomas. Recent cancer research led to a tumor stem cell paradigm stating that in certain tumors cells are hierarchically ordered with so-called tumor stem cells resembling the behavior of non-neoplastic stem cells. These cells might be accountable for specific cancer characteristics such as resistance to therapy and tumor recurrence.

Tumor stem cells (TSCs) have been identified in a variety of solid and hematologic neoplasms. Since TSCs are believed to harbor the ability to reconstitute neoplasms after therapy, identification and elimination of these cells are an indispensable part of novel therapeutic strategies against glioblastomas.

The aim of the present investigation is to identify glioblastoma stem cells depending on the expression of aldehyde dehydrogenase-1 (ALDH1) and to demonstrate their unique characteristics including neurosphere formation, colony formation and potential to differentiate into mature neural cell lines.

Therefore, established and primary glioblastoma cell lines obtained from freshly resected tumors are examined for the expression of ALDH1; the ability to grow to spheres under certain culturing conditions and the potential to differentiate to ma-

ture neural cell lines is correlated with ALDH1 expression. The functional impact of ALDH1 on neurosphere formation and clonogenicity is demonstrated in various inhibition experiments. The grade of neuronal differentiation in GBM cell lines in relation to ALDH1 expression is determined to evaluate the role of ALDH1 for the maintenance of an undifferentiated, stem cell-like cellular phenotype. Immunohistochemical stainings of resected glioblastomas are used to assess ALDH1 positivity in freshly obtained tumors. To evaluate the reliability of ALDH1 as a novel TSC marker in comparison to previously established indicators, flowcytometric analysis of CD133 and nestin expression is correlated with the results of the functional stem cell assays described in the present investigation.

3 Materials and methods

3.1 Antibodies

The primary antibodies used were anti-ALDH1 (monoclonal, IgG isotype, BD Biosciences, San Jose, CA, USA), anti-Musashi-1 (polyclonal, Cell Signaling Technology, Beverly, MA, USA), anti-Sox-2 (monoclonal, IgG, Millipore, Billerica, MA, USA), and anti-beta-III-tubulin (monoclonal, IgG isotype, Millipore).

3.2 Cell Culture

The human glioblastoma cell lines LN18 (Ishii et al., 1999), LN229 (Ishii et al., 1999)), LNZ308 (Ishii et al., 1999), and G139 (Schlegel et al., 2000) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Karlsruhe, Germany) under standard cell culture conditions at $37^{\circ}\mathrm{C}$ and 5% CO2. To investigate neurosphere formation, cells were grown in serum-free neurobasal medium consisting of Knockout-DMEM (Invitrogen), Nutrient Mixture Ham's F12 + L-glutamine (Invitrogen), and 10% Knockout-Serum Replacement (Invitrogen) with 1% N2-supplement (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1% natural mouse laminin (Invitrogen), 2 mM L-glutamine (Biochrom, Berlin, Germany), 100 U/ml penicillin (Biochrom), 100 μg/ml streptomycin (Biochrom), 10 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen), and 50 ng/ml epidermal growth factor (EGF) (human recombinant, Millipore). For non-adherent growth conditions, the culture dishes were covered with 0.1% gelatin type A (Sigma-Aldrich, St. Louis, MO, USA). For the ALDH1 inhibition experiments, retinoic acid and 4-diethylaminobenzaldehyde (Sigma-Aldrich) were added at a concentration of 100 μM every 48 hours together with fresh medium. The cells were grown in culture dishes until they began to form spheroids and were consequently transferred to culture flasks. Mouse embryonic stem cells (TBV2) were cultured as previously reported (Rudelius et al., 2003). For the limiting dilution assay, both LN18 and LN229 cells were plated onto 96-well plates at a

density of 10 cells/well and 1 cell/well, respectively. All cells were cultured in neurobasal medium with EGF and bFGF. ALDH1 inhibition was done as described above. After 12 days in culture, colonies were counted using a Zeiss Axiovert 25 microscope (Zeiss, Jena, Germany). A colony was defined as consisting of at least 20 cells. Multiple wells were counted for each cell density. The experiment was repeated twice for a total of three. For primary cell culture, resected glioblastoma specimens were obtained with patients' consent (according to the TUM medical faculty's guidelines for tissue preservation) from the Department of Neurosurgery (Head: Prof. Bernhard Meyer). Tumor tissue was mechanically dissociated with sterile scalpel blades and consequently enzymatically dissociated by 1-hour incubation in 2 mg/ml collagenase type I (Biochrom). Single-cell suspensions were cultured in supplemented DMEM and in neurobasal medium under standard cell culture conditions. Andrea Schäfer, Department of Neuropathology, Technical University Munich, performed the primary cell culture experiments in the context of her thesis. Parts of the results were generously provided for the current investigation.

3.3 Immunohistochemistry

Immunohistochemical analysis was performed on paraffin-embedded human tumor tissue, diagnosed as glioblastoma, grade IV according to the WHO classification. The tissue was sectioned 2 μm thick and deparaffinized. To unmask epitopes, tissue was boiled in citrate buffer (pH 6.0) for 7 minutes. After washing in TRIS buffer (pH 7.6) for 5 minutes, endogenous peroxidase quench was performed in 3% H_2O_2 for 15 minutes at room temperature, followed by blocking with avidin/biotin (Vector) for 15 minutes each. To block nonspecific binding sites, 5% goat serum (Normal, Dako) diluted in Dako REAL antibody solution was used. The anti-ALDH1 antibody was diluted in Dako REAL antibody solution (1:500). Antibody incubation was performed at $4\,^{\circ}\mathrm{C}$ using 200 μl per slide overnight. Detection was performed with the Dako REAL detection system, peroxidase/DAB+, rabbit/mouse. Slides were incubated for 15 minutes in a humidi-

fied chamber at room temperature with biotinylated secondary antibody and then with streptavidin peroxidase antibody for 15 minutes. Slides were incubated with DAB chromogen working solution for 4 minutes. Counterstaining was performed with Mayer's hemalaun. Julian Teufel, Department of Neuropathology, Technical University Munich, performed the immunohistochemical stainings in the context of his thesis. Parts of the results were generously provided for the current investigation.

3.4 Immunofluorescence

After cells were grown on microscope slides for 24 hours, they were washed in PBS (without Ca^{2+} or Mg^{2+}) followed by fixation in 4% formaldehyde/PBS for 30 minutes at room temperature. Cells were then incubated for 10 minutes with 0.25% Triton X-100/PBS and washed again in PBS. Blocking was performed for 30 minutes with 5% goat serum (Normal, Dako) diluted in Dako REAL antibody solution; cells were then incubated with primary antibodies for 1 hour (anti-ALDH1, 1:100) or 2 hours (anti-Sox-2, 1:100; anti-Musashi-1, 1:100) at room temperature. After the cells were washed with PBS, they were incubated with secondary antibody (FITC for ALDH1 and Sox2, 1:100; Cy-3 for Musashi, 1:100, Invitrogen) for 1 hour at room temperature and washed again. Nuclei were stained with DAPI (2 mg/ml) before mounting cells with VectaShield mounting medium (Vector Laboratories, Burlingame, CA, USA) and applying coverslips. Fluorescence images were captured with a Zeiss ApoTome epifluorescence confocal microscope equipped with a digital camera and acquisition software AxioVision LE Rel 4.5 (Zeiss).

3.5 Western Blotting

Cells were lysed in lysis buffer (New England Biolabs, Frankfurt, Germany) supplemented with 1 mM PMSF (Roth, Karlsruhe, Germany). Equal amounts of protein (10-20 μg) were separated by SDS-PAGE and transferred to Immobilion membranes (Millipore, Schwalbach, Germany). Unspecific binding sites were

blocked using 5% (w/v) non-fat dry milk in TBST (Tris-buffered saline Tween-20). Membranes were incubated with polyclonal primary antibodies diluted in TBST overnight at $4°C$. HRP-conjugated immunoglobulins (diluted 1:2000 in 5% non-fat dry milk/TBST) served as secondary antibodies and were probed for 1 hour at room temperature. Immunoreactivity was visualized by exposure to high-performance chemiluminescence film (Amersham, Amersham, UK) at the following antibody dilutions: anti-Sox-2, 1:500; anti-ALDH1, 1:500; anti-beta-III-tubulin, 1:1000; anti-Musashi-1, 1:500. Alpha-tubulin immunoblots served as protein loading controls (dilution 1:10.000).

3.6 Flow cytometry

The antibodies used were anti-CD133-PE (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-Nestin-APC (R&D Systems, Minneapolis, MN, USA). Flow cytometry was performed on a FACSCanto-II machine (BD Biosciences). For CD133 staining, cells were washed (washing buffer: Saponine in PBS (1:10)), trypsinized, centrifuged and resuspended in culture medium. Cells were counted (0.5 - 1 millions), again centrifuged and resuspended in PBS. Cells were then incubated in 50 μl staining buffer (anti-CD133-PE diluted 1:10 in PBS with 2 mM EDTA and 0.5% BSA) for 45 minutes at $4°C$. Cells were centrifuged, resuspended in 500 μl staining buffer and flow cytometry was performed. For Nestin staining, cells were washed with Saponin in PBS (1:10), trypsinized, centrifuged and resuspended in culture medium. Cells were counted (0.5 - 1 millions), again centrifuged and resuspended in PBS. After that, cells were incubated in 500 μl Paraformaldehyde (2%) for 15 minutes at $4°C$. 1 ml washing buffer was added, cells were centrifuged and resuspended in permeability buffer (Saponine in PBS (1:5)) for 15 minutes at room temperature. Again 1 ml washing buffer was added and cells were centrifuged. Consequently, cells were resuspended in 200 μl washing buffer with anti-Nestin-APC (dilution: 1:20) at $4°C$ for 1 hour. Then 1 ml washing buffer was added, cells were centrifuged, resuspended in 500 μl PBS wih 2 mM EDTA and 0.5% BSA and flow cytometry was performed. All flow cytometry experi-

ments were performed by Gero Brockhoff, MD, PhD, University of Regensburg, Germany.

3.7 Statistical analysis

One-way ANOVA was used to assess differences in average neurosphere size after ALDH1 inhibition. Statistical analysis was performed with Prism (GraphPad Software, La Jolla, CA, USA).

4 Results

4.1 Neurosphere-forming established glioblastoma cell lines show high levels of aldehyde dehydrogenase-1

To determine stem cell capacity, four established human glioblastoma cell lines (LN18, LN229, LNZ308, and G139) were screened for their ability to form neurospheres and for ALDH1 expression. Cells were grown under serum-free conditions in neurobasal medium supplemented with EGF and bFGF. Western blot analysis of the cell lines grown in supplemented DMEM showed a high expression of ALDH1 in LN18 cells and moderate expression in LNZ308 cells, whereas LN229 and G139 cells were negative for ALDH1 (Fig. 6). LN18 and LNZ308 cells formed neurospheres after 72 hours in culture (Fig. 7). Remarkably, spheroids built by LNZ308 cells were unstable and disintegrated at early stages of neurosphere formation. The ALDH1-negative cell lines LN229 and G139 did not form any spheroids under the same conditions (Fig. 6). In addition, the neurosphere-forming cell lines LN18 and LNZ308 showed co-expression of ALDH1 with Sox-2 and Musashi-1. Immunofluorescence staining was performed to confirm ALDH1 expression in LN18 cells. These cells were highly positive for ALDH1, and co-expression of Musashi-1 was demonstrated in double-staining experiments (Fig. 8A). Additionally, LN18 cells were positive for Sox-2. Stem cells derived from the murine embryonic stem cell line TBV2 served as positive controls for ALDH1, Musashi-1, and Sox-2.

4.2 Aldehyde dehydrogenase-1 inhibition impairs neurosphere formation and clonogenicity in established and primary glioblastoma cell lines

After identifying ALDH1 expression as a possible factor in the process of neurosphere formation, it was next investigated whether enzyme inhibition affects spheroid formation *in vitro*. Therefore LN18 cells and the primary cell line T15

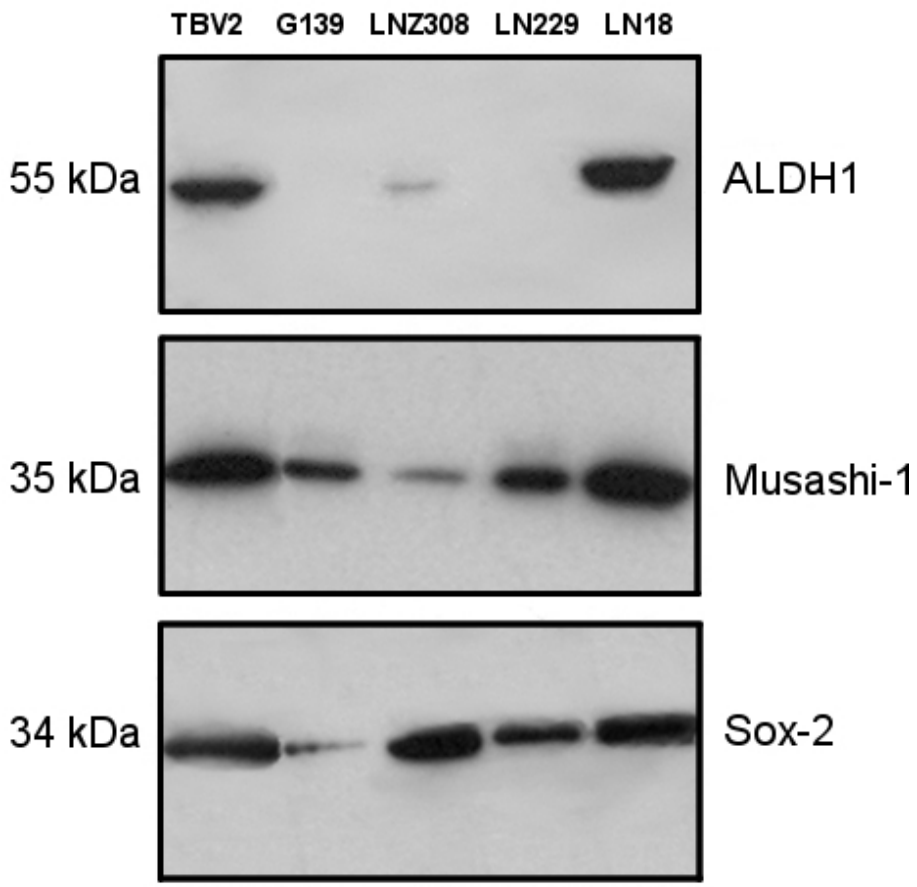

Figure 6: Western blot analysis shows high protein levels of ALDH1 in LN18 cells and in murine stem cells (TBV2) when grown in DMEM plus FCS. LNZ308 cells are moderately positive for ALDH1. ALDH1 co-expression with Musashi-1 and Sox-2 was found in LN18, LNZ308, and TBV2 cells. All three show neurosphere formation when cultured in neurobasal medium containing EGF and bFGF. G139 and LN229 are negative for ALDH1 and do not form spheroids under the same conditions. Figure published in Rasper et al., 2010, page 3.

were incubated under serum-free conditions in neurobasal medium with EGF and bFGF. For inhibition of ALDH1, 4-diethylaminobenzaldehyde (DEAB), retinoic acid, or a combination of both were added to the medium. DEAB and retinoic acid are specific inhibitors of ALDH1 (Elizondo et al., 2000, Chute et al., 2006). Untreated cells served as controls. The inhibitors did not affect cell viability com-

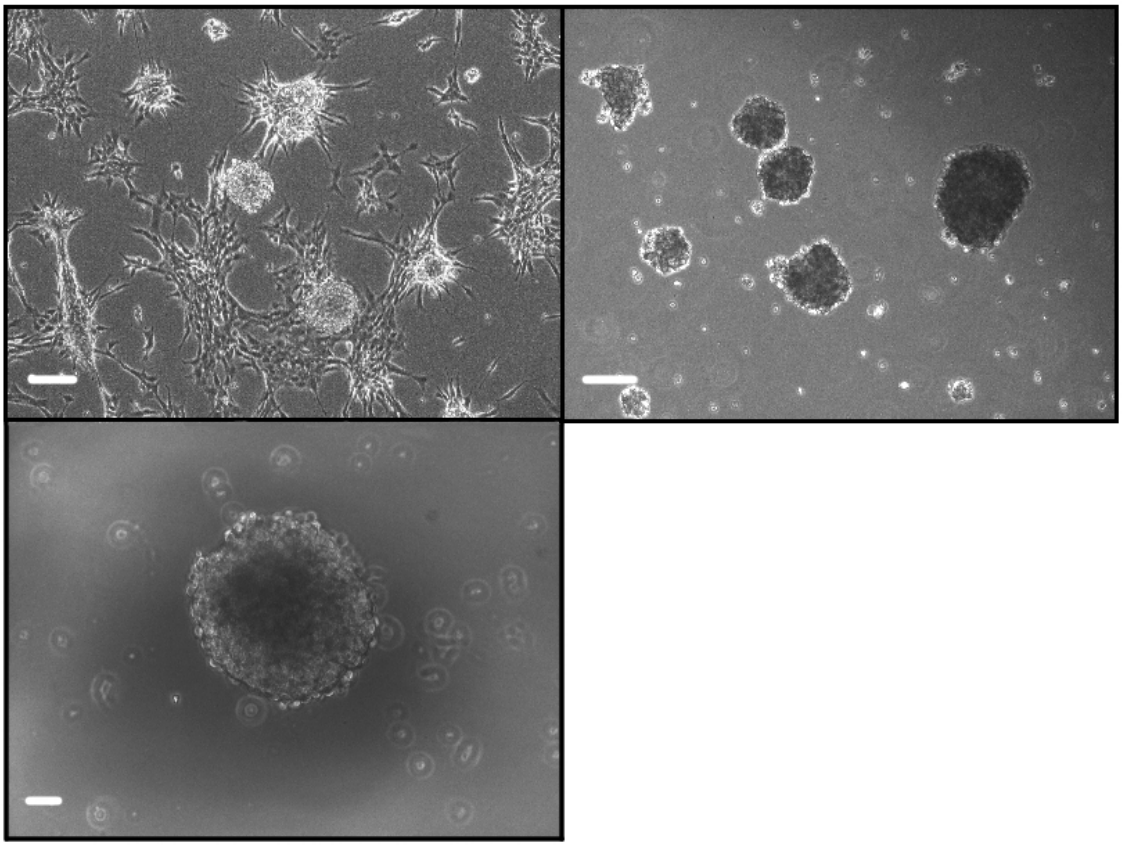

Figure 7: LN18 cells cultured in neurobasal medium. Aggregating cells after 24 hours (upper left), spheroids formed by aggregating cells still growing adherently after 72 hours (upper right); neurospheres detached from the ground of the culture dish after 96 hours (lower left). Scale bars represent 20 μm.

pared with untreated control cells (data not shown). After 14 days of cultivation, the inhibited cells had formed significantly fewer and smaller neurospheres than the controls (Fig. 9A and B). The combination of DEAB and retinoic acid led to the most significant decline in spheroid number and size. Because ALDH1 participates in the maintenance of an undifferentiated phenotype, the influence of ALDH1 inhibition on cell differentiation was analyzed. LN18 and T15 control cells showed moderate expression of beta-III-tubulin, a neuron-specific marker, when grown in neurobasal medium (Fig. 10). The inhibited cells were highly positive for beta-III-tubulin, reflecting a more differentiated state compared with untreated

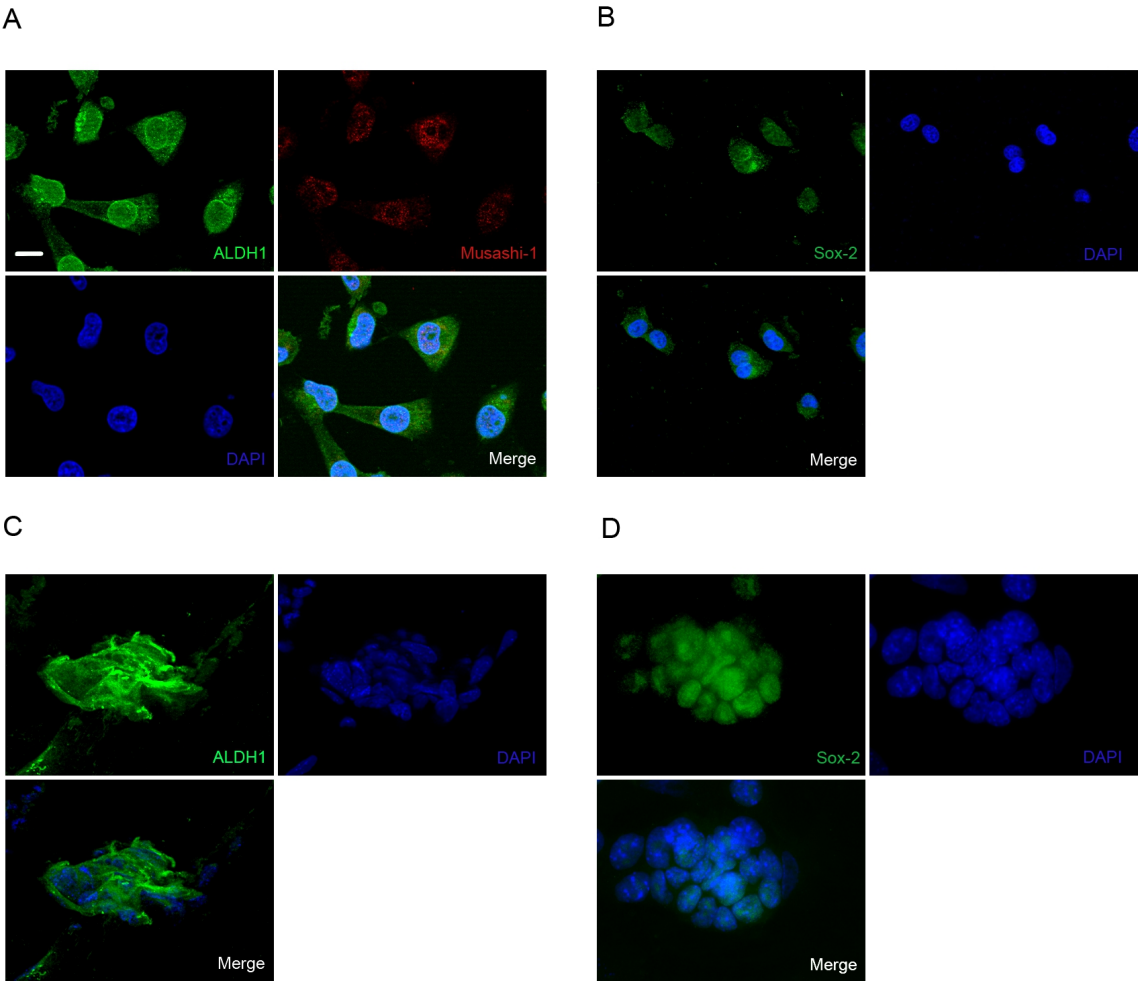

Figure 8: Immunofluorescence staining. (A) LN18 cells co-express ALDH1 (cytoplasmic) and Musashi-1 (nuclear) when cultured in DMEM plus FCS. (B) Additionally, the cells are positive for Sox-2 (nuclear). Murine stem cells (TBV2) serve as controls and are highly positive for both ALDH1 and Sox-2 (C and D). Nuclei stained with DAPI. Scale bar represents 10 μm. Figure published in Rasper et al., 2010, page 4.

controls. Murine embryonic stem cells (TBV2) were negative for the neuronal marker. ALDH1 inhibition did not induce differentiation in the ALDH1-negative cell lines LN229 and G139. To assess the clonogenic capacity of ALDH1-positive cells, LN18 and LN229 cells were plated onto 96-well plates at various densities, down to 1 cell/well. After 12 days of culture in neurobasal medium with EGF and bFGF, LN18 cells had formed colonies with an efficiency of 75.8% and neurospheres with a frequency of 18.9%. Even single-cell suspensions grew to colonies and neurospheres. Inhibition of ALDH1 in LN18 cells by retinoic acid reduced clonogenic capacity from 75.8% to 1.6%. The inhibited cells did not form spheroids. At least 10 ALDH1 negative LN229 cells per well were needed to obtain colonies with an efficiency of 4.3%. LN229 single-cell suspensions did not give rise to any colonies.

4.3 Aldehyde dehydrogenase-1 expression correlates negatively with the grade of neuronal differentiation in primary glioblastoma cultures

To evaluate the clinical relevance of ALDH1 expression in human GBM, the investigations were extended from the cell culture model to human tumor tissue. Cells obtained from freshly resected GBM specimens were cultured in supplemented DMEM and under serum-free conditions in neurobasal medium. All samples were collected from tumors diagnosed as high-grade astrocytomas (WHO grades III and IV). Western blot analysis showed that four primary cultures were highly positive for ALDH1, three had moderate levels, and two were negative when cultured in DMEM plus FCS (Fig. 11A). Beta-III-tubulin expression showed a reverse pattern, with cells with high levels of ALDH1 showing low expression of beta-III-tubulin and vice versa. Similar to established cell lines, primary cells partly differentiated when grown in neurobasal medium accompanied by expression of beta-III-tubulin (Fig. 11A and B). Western blot analysis of cells grown in DMEM without FCS confirmed that loss of ALDH1 expression and tendency to differentiate in neurobasal medium were not due to serum deprivation (data not

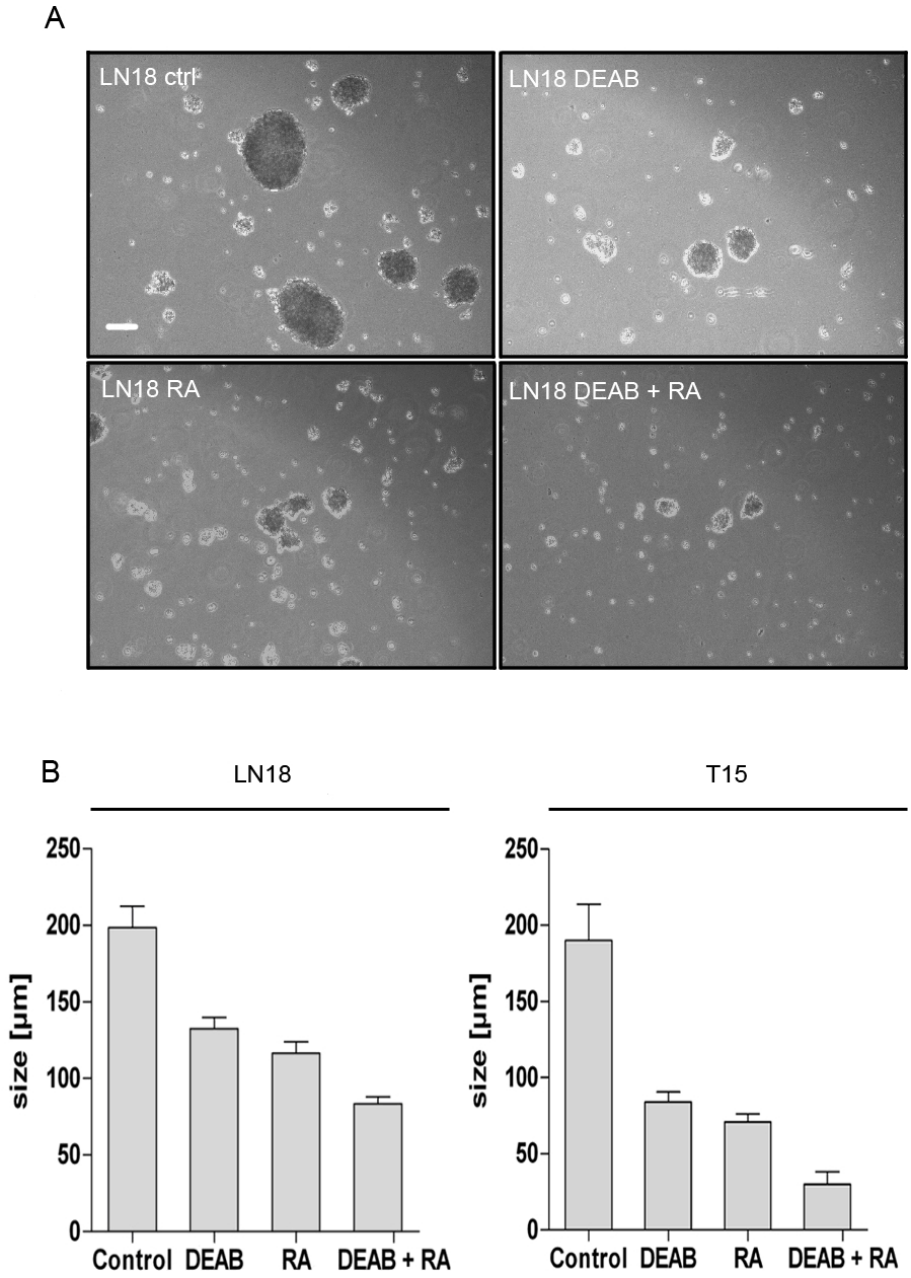

Figure 9: (A) Neurospheres built by LN18 cells after 14 days in culture (neurobasal medium). The inhibitors DEAB and retinoic acid (RA) were added at a concentration of 100 μM every 48 hours with fresh medium. Scale bar represents 50 μm. (B) Average neurosphere size after 14 days in culture. The spheres formed by inhibited cells were significantly smaller ($p<0.05$) than those built by LN18 and T15 control cells. Cells were treated with 100 μM DEAB, 100 μM retinoic acid, or a combination of both. Inhibition with DEAB and retinoic acid led to the most significant decline in neurosphere size. Figure published in Rasper et al., 2010, page 5.

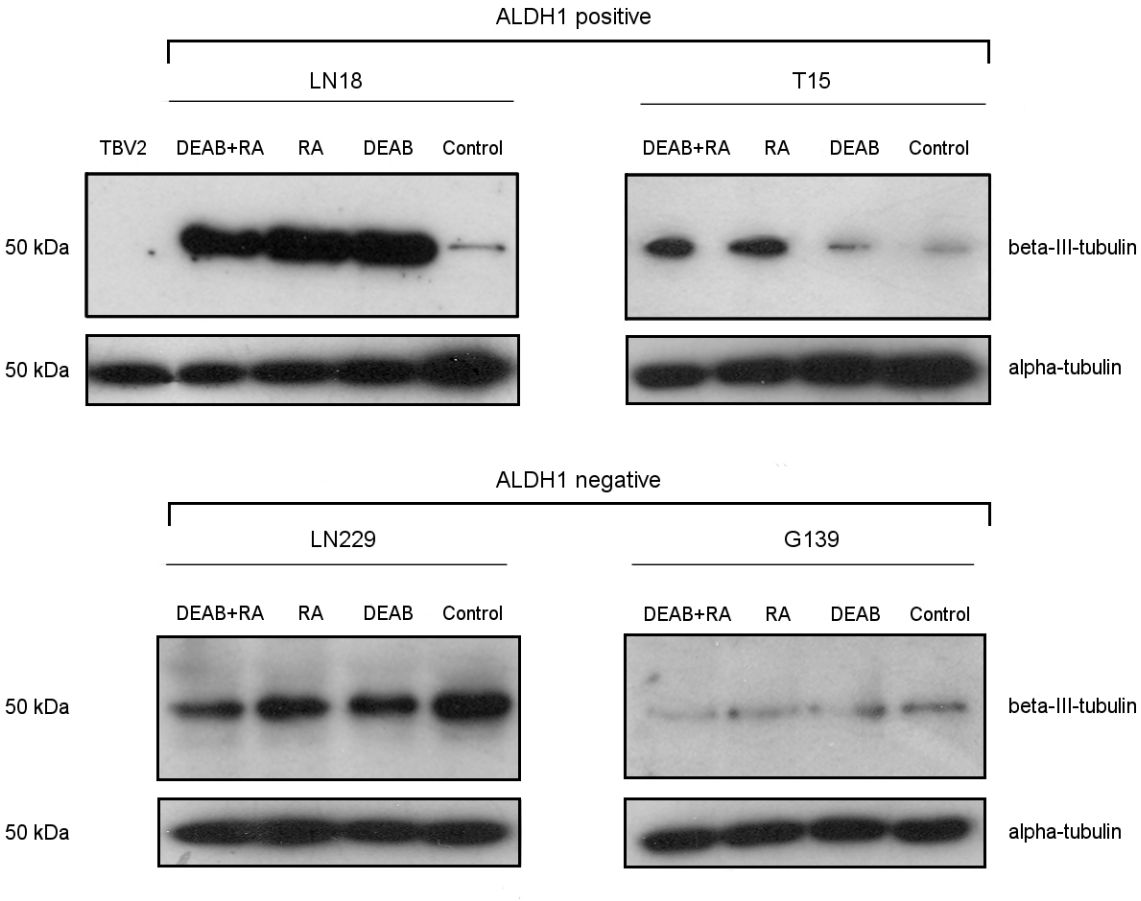

Figure 10: Expression of the neuron-specific marker beta-III-tubulin in LN18 cells and the primary cell line T15. All cells except for TBV2 were grown in neurobasal medium. LN18 and T15 control cells show moderate levels of beta-III-tubulin. Inhibition of ALDH1 induces pronounced differentiation, indicated by higher protein levels of beta-III-tubulin in both cell lines. The undifferentiated TBV2 stem cells are negative for beta-III-tubulin. ALDH1 inhibition does not induce differentiation in LN229 and G139 cells (ALDH1 negative). Alpha-tubulin was used as a protein loading control. Figure published in Rasper et al., 2010, page 6.

shown).

4.4 Immunohistochemistry shows aldehyde dehydrogenase-1 expression in resected glioblastomas and non-neoplastic neural stem cells

Immunohistochemical staining of paraffin sections from 24 resected glioblastomas was performed to confirm ALDH1 expression in tumor specimens. Various degrees of ALDH1 expression were found in 20 of the tumor samples, with four specimens negative. Interestingly, ALDH1-positive tumor cells were observed mainly in the vicinity of tumor vessels and in hypoxic areas adjacent to tumor necrosis (Fig. 12A). Immunohistochemical analysis on paraffin-embedded human subventricular zone (SVZ) was performed to confirm staining specificity. Several types of astrocytes, including multipotent stem cells, have been described as located in the human SVZ (Quinones-Hinojosa et al., 2006). ALDH1-positive cells were found in a scattered pattern within the subependymal ribbon (Fig. 12B). The brain tissue adjacent to the SVZ did not contain cells positive for ALDH1.

4.5 Unlike CD133 and nestin, aldehyde dehydrogenase-1 expression correlates with cancer stem cell capacity in functional assays

In order to investigate the role of nestin and CD133 in our cell lines, flow cytometry using specific antibodies was performed. The expression of nestin, a putative TSC marker, was analyzed. Both cell lines were found to be nearly a hundred percent positive for nestin (Fig. 13). The results for CD133 of the cell lines used in the present study are in accordance to recently published data doubting the validity of CD133 as a glioma TSC marker (Beier et al., 2007, Clement et al., 2009)). Both glioblastoma cell lines (LN18 and LN229) were negative for CD133 (Fig. 14). The colorectal cancer cell line HTC116 served as a positive control to rule out difficulties arising from staining faults. In conclusion neither CD133

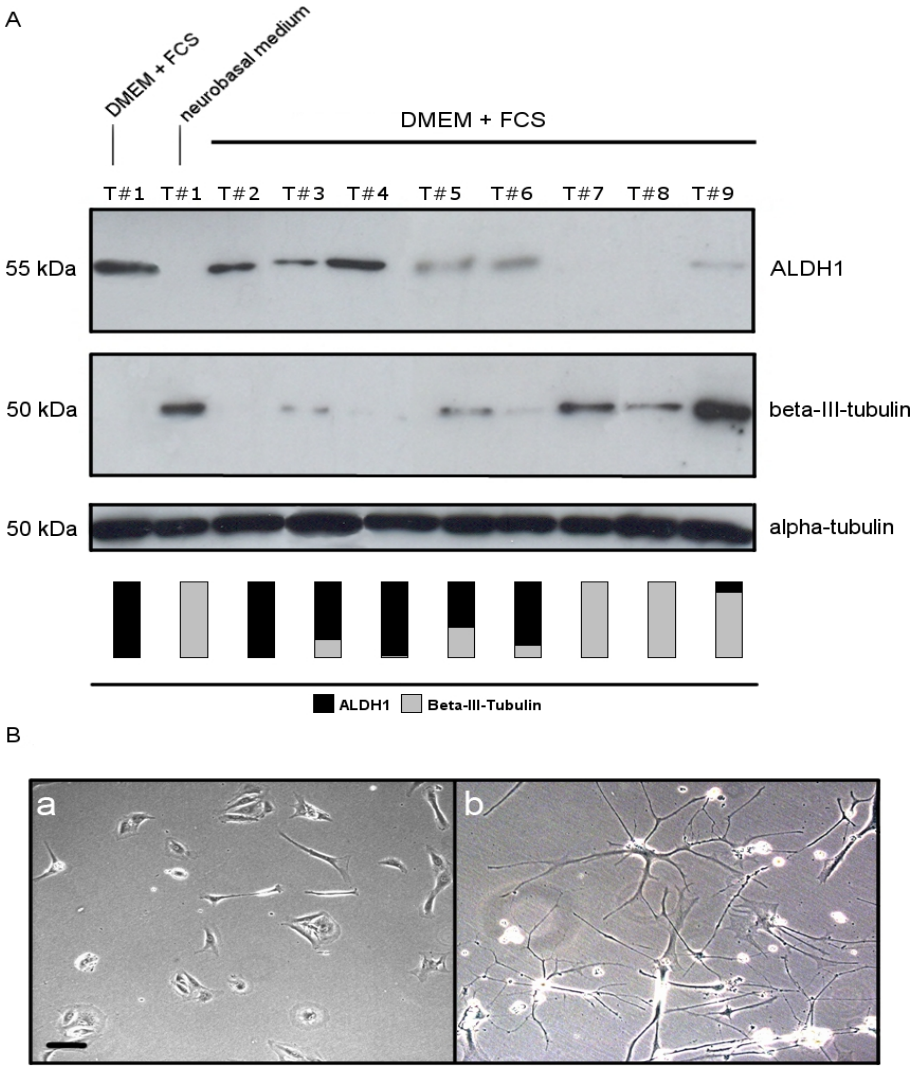

Figure 11: (A) ALDH1 and beta-III-tubulin expression in primary cultures obtained from fresh glioma samples (WHO grades III and IV) cultured in supplemented DMEM or neurobasal medium. ALDH1 and beta-III-tubulin expression in both media is exemplarily shown for tumor 1 (lane 2). Like the established cell line LN18, primary cell lines express high levels of ALDH1 in DMEM plus FCS and are negative when grown in neurobasal medium (and vice versa for beta-III-tubulin). All tumors are positive for either ALDH1 or beta-III-tubulin in DMEM plus FCS. The vertical bars visualize ALDH1 and beta-III-tubulin expression of each cell line. Alpha-tubulin serves as a protein loading control. (B) Tumor 1 grown in supplemented DMEM (a) and neurobasal medium (b). Tumor cells positive for ALDH1 under serum conditions differentiate into mature neural and glial cells when cultured in neurobasal medium. Scale bar represents 10 μm. Figure published in Rasper et al., 2010, page 7.

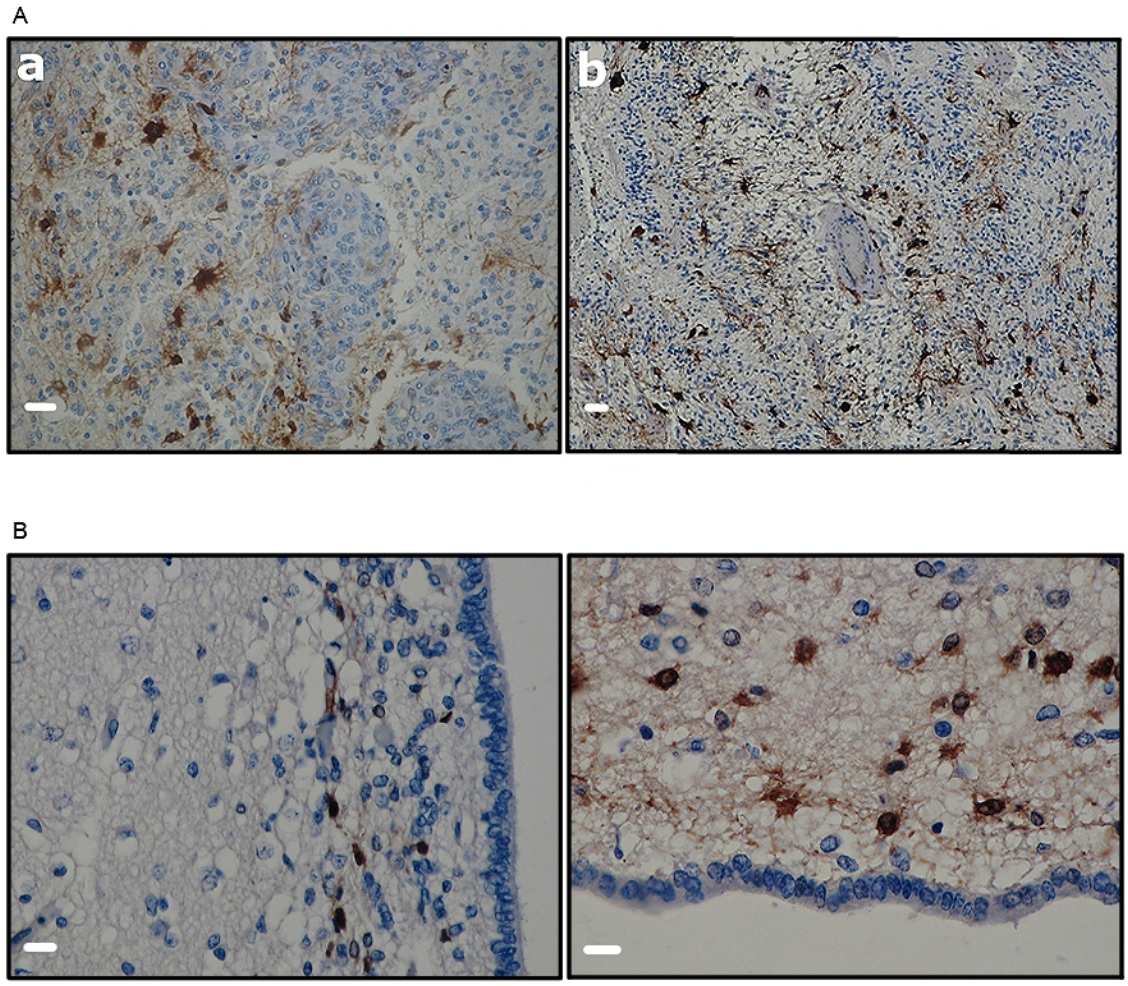

Figure 12: (A) Immunohistochemical staining showing tumor cells and proliferating vessels with ALDH1-positive cells (brown) in their vicinity (a). In (b), tumor necrosis, pseudo-palisades, and ALDH1-positive cells can be seen. (B) Staining of human subventricular zone (SVZ). Scattered ALDH1-positive cells are located in the subependymal ribbon. The brain tissue adjacent to the SVZ is negative for ALDH1. Scale bars represent 20 μm. Figure published in Rasper et al., 2010, page 8.

nor nestin expression correspond with the cells' behavior in functional TSC assays such as the neurosphere formation or limiting dilution assay described in the present investigation.

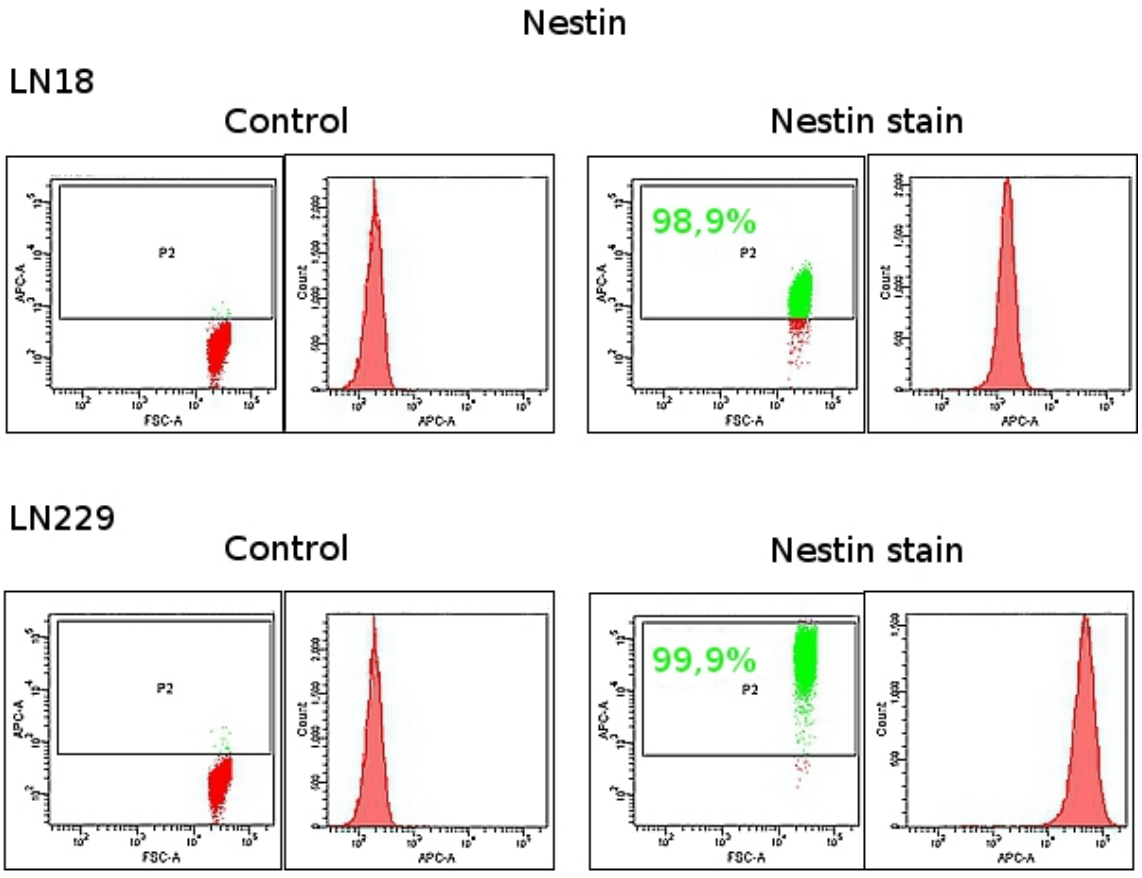

Figure 13: Flow cytometry of nestin expression in LN18 and LN229 cells. Both cell lines are nearly a hundred percent positive for nestin.

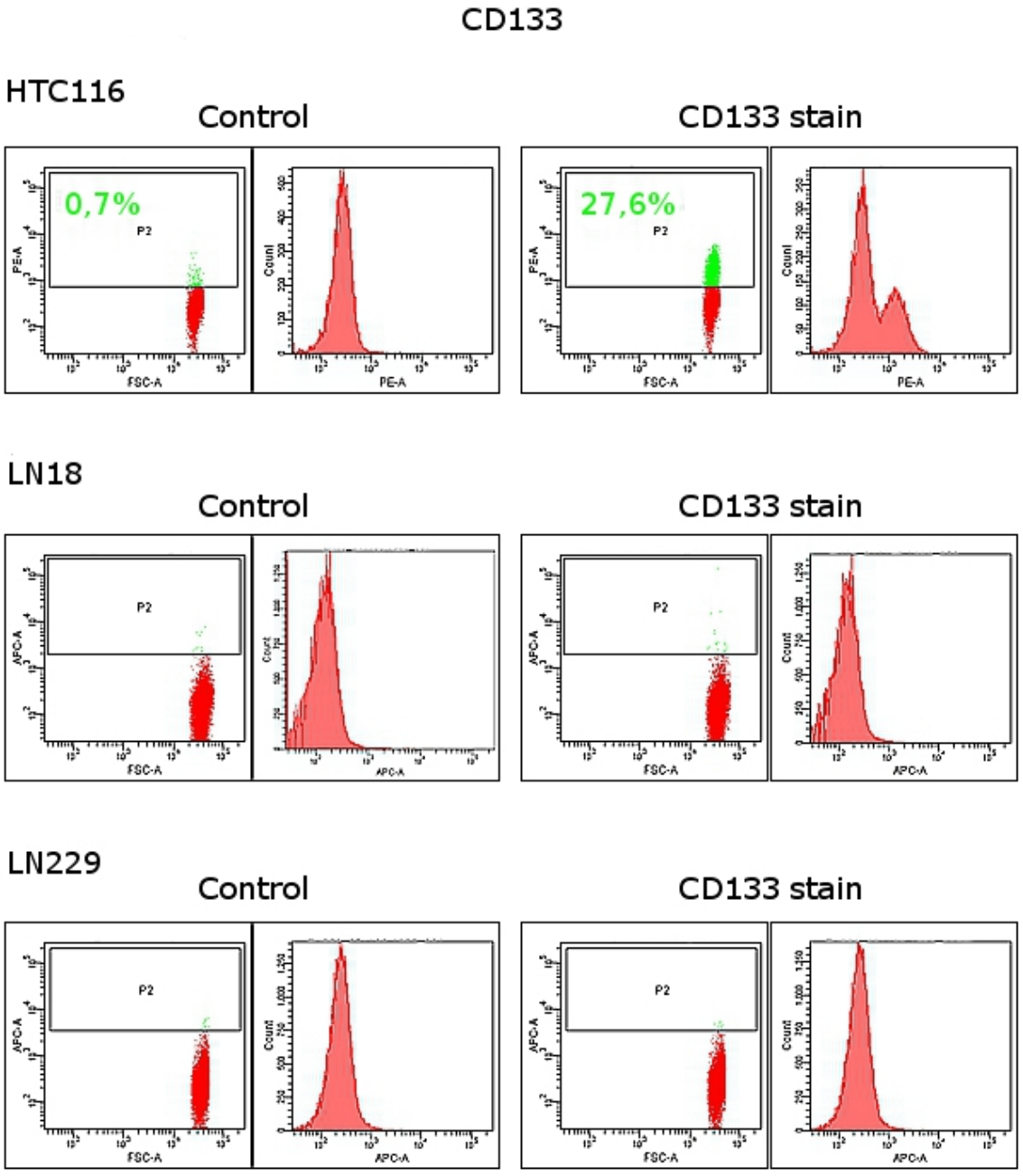

Figure 14: Flow cytometry of CD133 expression in LN18 and LN229 cells. Both cell lines are negative for CD133. HTC116 colorectal cancer cells serve as positive controls with 27.6% of cells showing expression of CD133.

5 Discussion

The poor clinical behavior and unfavorable prognosis of GBM could be due to the existence of a TSC subpopulation. TSCs have recently been shown as highly resistant to irradiation and chemotherapy and are likely the major source of tumor recurrence (Singh et al., 2004, Bao et al., 2006, Ricci-Vitiani et al., 2007, Frosina, 2009). Therefore identification and isolation of these cells seem crucial for a better understanding of tumor behavior, origin, and therapy. To further characterize TSCs in malignant glioma in the present investigation, established cell lines, primary cell cultures, and paraffin-embedded sections derived from human GBM were examined for the expression of ALDH1. As a member of the aldehyde dehydrogenase family, ALDH1 oxidates intracellular aldehydes and has been shown to catalyze the reaction of retinal to retinoic acid, a modulator of cell proliferation and differentiation (Chambon, 1996, Tocci et al., 1996, Zile, 2001, Collins, 2002, Sophos and Vasiliou, 2003, Zechel, 2005). Recently ALDH1 has been described as a marker for the identification of non-neoplastic stem cells and TSCs (Corti et al., 2006, Ginestier et al., 2007, Huang et al., 2009). In the current investigation, a correlation between the cellular protein level of ALDH1 and the ability to form neurospheres in human glioblastoma cells could be shown. It was demonstrated that established GBM lines harboring high levels of ALDH1 form tumor spheroids. ALDH1-negative cell lines did not build neurospheres when cultured under the same conditions. Moreover, inhibition of ALDH1 led to a significant decrease of neurosphere formation and clonogenic capacity. Inhibited cells expressed high levels of beta-III-tubulin, suggesting a higher grade of neuronal differentiation compared with untreated controls. High levels of ALDH1 in primary cultures obtained from freshly resected tumor tissue were observed, confirming that ALDH1 overexpression is not a phenomenon restricted to established cell lines. To date, cellular markers including CD133 have been used to identify TSCs in glioblastomas. Recently the relevance of CD133 as a reliable TSC marker was questioned, because it was shown that even CD133-negative GBM cells may behave as brain TSCs (Beier et al., 2007). The results of CD133 and nestin

flow cytometry in the present investigation are in the same line with these observations. In contrast to ALDH1 levels, CD133 and nestin expression did not correlate with tumor stem cell capacity observed in other TSC assays such as the neurosphere formation or colony formation assay. CD133 and nestin have been widely used to identify brain tumor stem cells; however, a functional role of these markers in the maintanance of a stem cell-like phenotype could never be demonstrated. The ALDH1 inhibition experiments described here suggest a vital character of ALDH1 expression for glioblastoma TSCs. ALDH1 inhibition impaired the cells' TSC characteristics including low grade of differentiation, neurosphere and colony formation. Considering the outstanding significance of GBM stem cells for therapy and patient survival, the identification of functional TSC markers such as ALDH1 might be an important step towards novel therapeutic strategies targeting at glioblastoma stem cells. The neurosphere formation assay is a well-established method to investigate functional properties of non-neoplastic neural stem cells and is also appropriate for the examination of brain TSCs. Unlike CD133 and nestin expression, neurosphere formation is a functional assay that investigates the effect of ALDH1 expression on stem cell characteristics *in vitro* and is therefore suitable for the ALDH1-inhibition experiments described in the current investigation.

5.1 ALDH1 and glioblastoma stem cells

Recently published data showed that ALDH1 characterizes non-neoplastic neural stem cells and TSCs in a variety of malignancies. Corti and colleagues isolated primitive neural stem cells from murine neurospheres via flow cytometry with Aldefluor®, a fluorescent substrate for ALDH1 (Corti et al., 2006). The ALDH1-positive cells showed self-renewal and neurosphere formation and could be differentiated into both neuronal and glial cells. Ginestier et al. used the Aldefluor® assay to sort normal mammary epithelium cells for their level of cytosolic ALDH1 (Ginestier et al., 2007). They found the capability to form spheroids (mammospheres) to be restricted to the Aldefluor®-positive cell population. Recently

ALDH1 has also been described as a stem cell marker in various solid neoplasms, including lung cancer (Jiang et al., 2009), breast carcinoma (Ginestier et al., 2007), and colorectal cancer (Huang et al., 2009). The present investigation showed that glioblastoma cell lines harboring high levels of ALDH1 form neurospheres, suggesting stem cell capacity. To date, ALDH1 expression has been found to characterize non-neoplastic neural stem cells as well as malignant stem cells in various solid tumors; the current work suggests that ALDH1 as a TSC marker in human glioblastoma. It seems that high ALDH1 activity in GBM cells is involved in the maintenance of an undifferentiated stem cell-like phenotype. LN18 cells grown in DMEM plus FCS had high levels of ALDH1 and were negative for beta-III-tubulin, suggesting an undifferentiated state. Primary cell cultures highly positive for ALDH1 showed low levels of beta-III-tubulin and vice versa. None of the established cell lines built spheroids when grown in supplemented DMEM. When transferred to neurobasal medium, neurospheres were formed only by the two cell lines (LN18 and LNZ308) that showed ALDH1 expression in DMEM plus FCS. It is therefore likely that ALDH1 overexpression characterizes TSCs that can initiate neurosphere formation. Under serum conditions, these cells remain in a proliferative and undifferentiated state. Culturing in neurobasal medium partly pushes the TSCs into differentiation, as indicated by a decrease of ALDH1 expression and a moderate increase in beta-III-tubulin level. Beta-III-tubulin is a neural marker appearing at early stages of neuronal differentiation as well as in mature neurons (Fanarraga et al., 1999). Consequently, low levels of beta-III-tubulin expression do not necessarily reflect mature neuronal differentiation. Multipotent ALDH1-positive stem or progenitor cells might therefore remain in an undifferentiated and replicative state when cultured in DMEM plus FCS. Neurobasal medium supplemented with EGF and bFGF promotes the stem cell phenotype, including asymmetrical division, neurosphere formation, and neural differentiation. Inhibition of ALDH1 in LN18 and T15 cells led to pronounced differentiation with high expression of the neuron-specific marker. This effect was not seen in ALDH1-negative cell lines, suggesting a lack of multipotency and ability to differentiate. It might be hypothesized that inhibition of ALDH1 leads to a

premature differentiation of TSCs and thus to an adverse effect on neurosphere formation. The clonogenic capacity of ALDH1-positive cells could be demonstrated by limiting dilution assays. Even single LN18 cells formed both colonies and neurospheres, whereas single LN229 cells did not show any proliferation in stem cell-promoting medium. ALDH1 inhibition significantly impaired the clonogenic and neurosphere formation capacity of LN18 cells, supporting the essential role of ALDH1 for the maintenance of a stem cell-like cell phenotype.

5.2 Functional implications of ALDH1 expression

Little is known about the functional role of ALDH1 in TSCs and in the process of neurosphere formation. The enzyme catalyzes the oxidation of retinal to retinoic acid, which is known to induce and regulate various biological effects. Mediated by the retinoic acid receptor (RAR) and the retinoid X receptor (RXR), it affects development, cell division, apoptosis, and differentiation (Chambon, 1996, Tocci et al., 1996, Zile, 2001, Collins, 2002, Zechel, 2005). Zeng and colleagues recently showed that treatment of an established glioblastoma cell line with retinoic acid leads to upregulation of genes responsible for apoptosis and differentiation (Zeng et al., 2009). Moreb et al. treated lung cancer cell lines with all-trans retinoic acid, causing a significant decrease in ALDH1 enzyme activity and protein level (Moreb et al., 2005). Considering these observations and the inhibition experiments described here, it seems most likely that high ALDH1 activity is involved in the maintenance of an undifferentiated, stem cell-like phenotype. In the current study, neurospheres were formed by cell lines that co-express ALDH1 with the formerly described TSC markers Sox-2 and Musashi-1. Cell lines that are positive for Musashi-1 and Sox-2 but negative for ALDH1 (LN229 and G139) do not form spheroids. It might be therefore hypothesized that ALDH1 expression in combination with Musashi-1 and Sox-2 facilitates neurosphere formation in established GBM cell lines. To confirm ALDH1 expression in patient specimens, immunohistochemical analysis of glioblastoma samples was performed. In many tumors, ALDH1-positive cells were found in the vicinity of tumor vessels or around

necrotic areas (Fig. 7A). Calabrese et al. hypothesized GBM TSCs to be predominantly localized in the proximity of tumor vessels, showing close interaction with endothelial cells (Calabrese et al., 2007). Hypoxia has been claimed to attract neural stem cells in intracranial glioma xenografts (Zhao et al., 2008). Li et al. demonstrated that, in response to hypoxia, glioma stem cells express hypoxia-inducible factors (HIFs) that induce tumor angiogenesis (Li et al., 2009). In addition, HIF2α was found to be co-localized with TSC markers in tumor specimens. ALDH1 is an enzyme with detoxifying capacity and could therefore be involved in the protection of TSCs against cellular stress in hypoxic areas. This might explain why ALDH1-positive tumor cells were oftentimes found adjacent to necrotic areas. Magni et al. demonstrated that the detoxifying capacity of ALDH1 facilitates cell resistance against alkylating agents such as cyclophosphamide (Magni et al., 1996). This observation has clinical implications for glioblastoma therapy. The standard treatment of newly diagnosed GBM currently consists of surgical resection and temozolomide given concomitantly with and after radiotherapy (Stupp et al., 2005). Glioblastoma stem cells harboring high levels of ALDH1 could be resistant to this alkylating agent, thus escaping chemotherapy and causing tumor relapse.

5.3 ALDH1 in non-neoplastic neural stem and progenitor cells

Immunohistochemical analysis of paraffin-embedded human SVZ showed specific staining of ALDH1-positive cells within the subependymal ribbon. This area contains a small subpopulation of SVZ astrocytes that behave as multipotent stem cells (Quinones-Hinojosa et al., 2006). Morphologically, these cells can hardly be distinguished from other cell types present in the SVZ such as neuronal precursor cells, other types of astrocytes, or oligodendrocytes. Consequently, ALDH1-positive SVZ astrocytes cannot definitely be categorized as stem cells. However, taking into account the finding that normal neural stem cells were identified by their expression of ALDH1 (Corti et al., 2006), it seems that these ALDH1-positive SVZ cells are stem or progenitor cells. In conclusion, the data of the present in-

vestigation suggest that ALDH1 is a novel TSC marker in human glioblastoma. As a cytosolic protein it is easily detectable, and cells can be effectively sorted for their ALDH1 expression using flow cytometry. In addition, ALDH1 could be an attractive target of advanced antitumor therapies against human malignant glioma. This work suggests various effects of ALDH1 expression on GBM stem cells. High enzyme levels seem to keep TSCs in an undifferentiated state, facilitate neurosphere formation and clonogenicity. Further research is needed for an advanced understanding of the impact of ALDH1 on these mechanisms. Tumor stem cells are presumably responsible for resistance to therapy and for disease relapse. Consequently, the effective treatment of the TSC subfraction within glioblastomas is likely to be an indispensable part of novel therapeutic strategies against this devastating disease.

6 Conclusion

Glioblastoma is the most common and most aggressive primary brain tumor in adults accounting for approximately 40% of all glial tumors (Preston-Martin, 1996). Accompanied by an unfavorable prognosis, tumor relapse occurs regularly despite surgical resection, irradiation and chemotherapy. The tendency to recurr after treatment might be due to the existence of a still poorly characterized tumor stem cell subfraction in glioblastoma. Due to the tumor stem cell paradigm, solid and hematologic neoplasms show a cellular hierarchy; the cancer stem cell subpopulation bears all capacities to reconstitute the tumor mass after therapy: pluripotency, proliferation and resistance to chemo- and irradiation therapy. Identification, characterization and treatment of this cellular subfraction seem therefore crucial for further insight into tumor biology and for the development of novel antitumor therapies. In the past efforts have been made to find cellular markers for the identification of CSCs in glioblastoma. Besides nestin, CD133 has long remained the most common and accepted marker of stemness in glioblastoma cells. Nevertheless, the significance of CD133 as a CSC marker has recently been doubted since it could be shown that even CD133 negative GBM cells can

harbor brain tumor stem cell characteristics (Beier et al., 2007, Clement et al., 2009). CD133 and nestin have been widely used in glioma stem cell studies; however, the functional role of these markers for brain tumor stem cells has never been determined. Consequently, the discovery of novel and reliable glioblastoma TSC markers with a functional impact on stem cell biology is of great importance. The results of the present investigation suggest that overexpression of the enzyme aldehyde dehydrogenase-1 is associated with tumor stem cell capacity in glioblastoma cells. Using the neurosphere formation assay as a functional method to identify brain tumor stem cells it was shown that high levels of ALDH1 facilitate neurosphere formation in established glioblastoma cell lines. ALDH1-negative cells did not form spheroids under the same culturing conditions. Even single ALDH1 positive cells gave rise to colonies and neurospheres suggesting stem cell capacity. The functional role of ALDH1 in TSC biology was demonstrated in various inhibition experiments. ALDH1 inhibition *in vitro* decreased both the number and size of neurospheres. In addition, ALDH1 inhibition depleted cells of their clonogenic capacity. High levels of ALDH1 seemed to keep tumor cells in an undifferentiated, stem cell-like state indicated by low expression of the neuron-specific marker beta-III-tubulin; ALDH1 inhibition in contrast induced premature differentiation. Flow cytometry showed that cellular ALDH1 levels, but not CD133 or nestin expression corresponded with the results of *in vitro* tumor stem assays such as the neurosphere or colony formation assay.

References

Balss, J., Meyer, J., Mueller, W., Korshunov, A., Hartmann, C., von Deimling, A. Analysis of the idh1 codon 132 mutation in brain tumors. Acta Neuropathol 116 (2008) 597–602.

Bao, S., Wu, Q., McLendon, R. E., Hao, Y., Shi, Q., Hjelmeland, A. B., Dewhirst, M. W., Bigner, D. D., Rich, J. N. Glioma stem cells promote radioresistance by preferential activation of the dna damage response. Int J Epidemiol 444 (2006) 756–60.

Beier, D., Hau, P., Proescholdt, M., Lohmeier, A., Wischhusen, J., Oefner, P. J., Aigner, L., Brawanski, A., Bogdahn, U., Beier, C. P. Cd133(+) and cd133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. Cancer Res 67 (2007) 4010–5.

Bondy, M. L., Scheurer, M. E., Malmer, B., Barnholtz-Sloan, J. S., Davis, F. G., Il'yasova, D., Kruchko, C., McCarthy, B. J., Rajaraman, P., Schwartzbaum, J. A., Sadetzki, S., Schlehofer, B., Tihan, T., Wiemels, J. L., Wrensch, M., Buffler, P. A. Brain tumor epidemiology: consensus from the brain tumor epidemiology consortium. Cancer 113 (2008) 1953–68.

Bonnet, D., Dick, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3 (1997) 730–7.

Brooks, P. J., Theruvathu, J. A. Dna adducts from acetaldehyde: implications for alcohol-related carcinogenesis. Alcohol 35 (2005) 187–93.

Brown, M., Schrot, R., Bauer, K., Letendre, D. Incidence of first primary central nervous system tumors in california, 2001-2005. J Neurooncol 94 (2009) 249–61.

Calabrese, C., Poppleton, H., Kocak, M., Hogg, T. L., Fuller, C., Hamner, B., Oh, E. Y., Gaber, M. W., Finklestein, D., Allen, M., Frank, A., Bayazitov, I. T., Zakharenko, S. S., Gajjar, A., Davidoff, A., Gilbertson, R. J. A perivascular niche for brain tumor stem cells. Cancer Cell 11 (2007) 69–82.

Chambon, P. A decade of molecular biology of retinoic acid receptors. Faseb J 10 (1996) 940–54.

Chute, J. P., Muramoto, G. G., Whitesides, J., Colvin, M., Safi, R., Chao, N. J., McDonnell, D. P. Inhibition of aldehyde dehydrogenase and retinoid signaling induces the expansion of human hematopoietic stem cells. Proc Natl Acad Sci U S A 103 (2006) 11707–12.

Clement, V., Dutoit, V., Marino, D., Dietrich, P. Y., Radovanovic, I. Limits of cd133 as a marker of glioma self-renewing cells. Int J Cancer 125 (2009) 244–8.

Collins, S. J. The role of retinoids and retinoic acid receptors in normal hematopoiesis. Leukemia 16 (2002) 1896–905.

Corti, S., Locatelli, F., Papadimitriou, D., Donadoni, C., Salani, S., Del Bo, R., Strazzer, S., Bresolin, N., Comi, G. P. Identification of a primitive brain-derived neural stem cell population based on aldehyde dehydrogenase activity. Stem Cells 24 (2006) 975–85.

Counsell, C. E., Grant, R. Incidence studies of primary and secondary intracranial tumors: a systematic review of their methodology and results. J Neurooncol 37 (1998) 241–50.

Dahlstrand, J., Collins, V. P., Lendahl, U. Expression of the class vi intermediate filament nestin in human central nervous system tumors. Cancer Res 52 (1992) 5334–41.

Elizondo, G., Corchero, J., Sterneck, E., Gonzalez, F. J. Feedback inhibition of the retinaldehyde dehydrogenase gene aldh1 by retinoic acid through retinoic acid receptor alpha and ccaat/enhancer-binding protein beta. J Biol Chem 275 (2000) 39747–53.

Fanarraga, M. L., Avila, J., Zabala, J. C. Expression of unphosphorylated class iii beta-tubulin isotype in neuroepithelial cells demonstrates neuroblast commitment and differentiation. Eur J Neurosci 11 (1999) 517–527.

Frosina, G. Dna repair and resistance of gliomas to chemotherapy and radiotherapy. Mol Cancer Res 7 (2009) 989–99.

Ginestier, C., Hur, M. H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., Jacquemier, J., Viens, P., Kleer, C. G., Liu, S., Schott, A., Hayes, D., Birnbaum, D., Wicha, M. S., Dontu, G. Aldh1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 1 (2007) 555–67.

Gravendeel, L. A. M., Kloosterhof, N. K., Bralten, L. B. C., van Marion, R., Dubbink, H. J., Dinjens, W., Bleeker, F. E., Hoogenraad, C. C., Michiels, E., Kros, J. M., van den Bent, M., Smitt, P. A. E. S., French, P. J. Segregation of non-p.r132h mutations in idh1 in distinct molecular subtypes of glioma. Hum Mutat 31 (2010) E1186–E1199.

Hegi, M. E., Diserens, A.-C., Gorlia, T., Hamou, M.-F., de Tribolet, N., Weller, M., Kros, J. M., Hainfellner, J. A., Mason, W., Mariani, L., Bromberg, J. E. C., Hau, P., Mirimanoff, R. O., Cairncross, J. G., Janzer, R. C., Stupp, R. Mgmt gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 352 (2005) 997–1003.

Huang, E. H., Hynes, M. J., Zhang, T., Ginestier, C., Dontu, G., Appelman, H., Fields, J. Z., Wicha, M. S., Boman, B. M. Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (sc) and tracks sc overpopulation during colon tumorigenesis. Cancer Res 69 (2009) 3382–9.

Ishii, N., Maier, D., Merlo, A., Tada, M., Sawamura, Y., Diserens, A. C., Van Meir, E. G. Frequent co-alterations of tp53, p16/cdkn2a, p14arf, pten tumor suppressor genes in human glioma cell lines. Brain Pathol 9 (1999) 469–79.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Thun, M. J. Cancer statistics, 2009. CA Cancer J Clin 59 (2009) 225–49.

Jiang, F., Qiu, Q., Khanna, A., Todd, N. W., Deepak, J., Xing, L., Wang, H., Liu,

Z., Su, Y., Stass, S. A., Katz, R. L. Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. Mol Cancer Res 7 (2009) 330–8.

Jones, R. J., Barber, J. P., Vala, M. S., Collector, M. I., Kaufmann, S. H., Ludeman, S. M., Colvin, O. M., Hilton, J. Assessment of aldehyde dehydrogenase in viable cells. Blood 85 (1995) 2742–6.

Komitova, M., Eriksson, P. S. Sox-2 is expressed by neural progenitors and astroglia in the adult rat brain. Neurosci Lett 369 (2004) 24–7.

Li, Z., Bao, S., Wu, Q., Wang, H., Eyler, C., Sathornsumetee, S., Shi, Q., Cao, Y., Lathia, J., McLendon, R. E., Hjelmeland, A. B., Rich, J. N. Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells. Cancer Cell 15 (2009) 501–13.

Louis, D. N., Ohgaki, H., Wiestler, O. D., Cavenee, W. K., Burger, P. C., Jouvet, A., Scheithauer, B. W., Kleihues, P. The 2007 who classification of tumours of the central nervous system. Acta Neuropathol 114 (2007) 97–109.

Ma, Y. H., Mentlein, R., Knerlich, F., Kruse, M. L., Mehdorn, H. M., Held-Feindt, J. Expression of stem cell markers in human astrocytomas of different who grades. J Neurooncol 86 (2008) 31–45.

Magni, M., Shammah, S., Schiro, R., Mellado, W., Dalla-Favera, R., Gianni, A. M. Induction of cyclophosphamide-resistance by aldehyde-dehydrogenase gene transfer. Blood 87 (1996) 1097–103.

Marchitti, S. A., Brocker, C., Stagos, D., Vasiliou, V. Non-p450 aldehyde oxidizing enzymes: the aldehyde dehydrogenase superfamily. Expert Opin Drug Metab Toxicol 4 (2008) 697–720.

Moreb, J. S., Gabr, A., Vartikar, G. R., Gowda, S., Zucali, J. R., Mohuczy, D. Retinoic acid down-regulates aldehyde dehydrogenase and increases cytotoxicity of 4-hydroperoxycyclophosphamide and acetaldehyde. J Pharmacol Exp Ther 312 (2005) 339–45.

Neglia, J. P., Robison, L. L., Stovall, M., Liu, Y., Packer, R. J., Hammond, S., Yasui, Y., Kasper, C. E., Mertens, A. C., Donaldson, S. S., Meadows, A. T., Inskip, P. D. New primary neoplasms of the central nervous system in survivors of childhood cancer: a report from the childhood cancer survivor study. J Natl Cancer Inst 98 (2006) 1528–37.

Ohgaki, H., Kleihues, P. Population-based studies on incidence, survival rates, and genetic alterations in astrocytic and oligodendroglial gliomas. J Neuropathol Exp Neurol 64 (2005) 479–89.

Ohgaki, H., Kleihues, P. Genetic alterations and signaling pathways in the evolution of gliomas. Cancer Sci 100 (2009) 2235–41.

Okano, H., Kawahara, H., Toriya, M., Nakao, K., Shibata, S., Imai, T. Function of rna-binding protein musashi-1 in stem cells. Exp Cell Res 306 (2005) 349–56.

Olsson, M., Lindahl, T. Repair of alkylated dna in escherichia coli. methyl group transfer from o6-methylguanine to a protein cysteine residue. J Biol Chem 255 (1980) 10569–10571.

Pappa, A., Brown, D., Koutalos, Y., DeGregori, J., White, C., Vasiliou, V. Human aldehyde dehydrogenase 3a1 inhibits proliferation and promotes survival of human corneal epithelial cells. J Biol Chem 280 (2005) 27998–8006.

Pegg, A. E., Wiest, L., Foote, R. S., Mitra, S., Perry, W. Purification and properties of o6-methylguanine-dna transmethylase from rat liver. J Biol Chem 258 (1983) 2327–2333.

Preston-Martin, S. Epidemiology of primary cns neoplasms. Neurol Clin 14 (1996) 273–90.

Quinones-Hinojosa, A., Sanai, N., Soriano-Navarro, M., Gonzalez-Perez, O., Mirzadeh, Z., Gil-Perotin, S., Romero-Rodriguez, R., Berger, M. S., Garcia-Verdugo, J. M., Alvarez-Buylla, A. Cellular composition and cytoarchitecture

of the adult human subventricular zone: a niche of neural stem cells. J Comp Neurol 494 (2006) 415–34.

Reya, T., Morrison, S. J., Clarke, M. F., Weissman, I. L. Stem cells, cancer, and cancer stem cells. Nature 414 (2001) 105–11.

Reynolds, B. A., Weiss, S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255 (1992) 1707–10.

Ricci-Vitiani, L., Lombardi, D. G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C., De Maria, R. Identification and expansion of human colon-cancer-initiating cells. Nature 445 (2007) 111–5.

Rudelius, M., Daldrup-Link, H. E., Heinzmann, U., Piontek, G., Settles, M., Link, T. M., Schlegel, J. Highly efficient paramagnetic labelling of embryonic and neuronal stem cells. Eur J Nucl Med Mol Imaging 30 (2003) 1038–44.

Ruder, A. M., Waters, M. A., Butler, M. A., Carreon, T., Calvert, G. M., Davis-King, K. E., Schulte, P. A., Sanderson, W. T., Ward, E. M., Connally, L. B., Heineman, E. F., Mandel, J. S., Morton, R. F., Reding, D. J., Rosenman, K. D., Talaska, G. Gliomas and farm pesticide exposure in men: the upper midwest health study. Arch Environ Health 59 (2004) 650–7.

Schlegel, J., Piontek, G., Budde, B., Neff, F., Kraus, A. The akt/protein kinase b-dependent anti-apoptotic pathway and the mitogen-activated protein kinase cascade are alternatively activated in human glioblastoma multiforme. Cancer Lett 158 (2000) 103–8.

Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J., Dirks, P. B. Identification of a cancer stem cell in human brain tumors. Cancer Res 63 (2003) 5821–8.

Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., Henkelman, R. M., Cusimano, M. D., Dirks, P. B. Identification of human brain tumour initiating cells. Nature 432 (2004) 396–401.

Sladek, N. E. Aldehyde dehydrogenase-mediated cellular relative insensitivity to the oxazaphosphorines. Curr Pharm Des 5 (1999) 607–25.

Sophos, N. A., Vasiliou, V. Aldehyde dehydrogenase gene superfamily: the 2002 update. Chem Biol Interact 143-144 (2003) 5–22.

Stupp, R., Mason, W. P., van den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J., Belanger, K., Brandes, A. A., Marosi, C., Bogdahn, U., Curschmann, J., Janzer, R. C., Ludwin, S. K., Gorlia, T., Allgeier, A., Lacombe, D., Cairncross, J. G., Eisenhauer, E., Mirimanoff, R. O. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352 (2005) 987–96.

Suzuki, H., Taguchi, T., Tanaka, H., Kataoka, H., Li, Z., Muramatsu, K., Gondo, T., Kawai, S. Neurospheres induced from bone marrow stromal cells are multipotent for differentiation into neuron, astrocyte, and oligodendrocyte phenotypes. Biochem Biophys Res Commun 322 (2004) 918–922.

Tocci, A., Parolini, I., Gabbianelli, M., Testa, U., Luchetti, L., Samoggia, P., Masella, B., Russo, G., Valtieri, M., Peschle, C. Dual action of retinoic acid on human embryonic/fetal hematopoiesis: blockade of primitive progenitor proliferation and shift from multipotent/erythroid/monocytic to granulocytic differentiation program. Blood 88 (1996) 2878–88.

Tohyama, T., Lee, V. M., Rorke, L. B., Marvin, M., McKay, R. D., Trojanowski, J. Q. Nestin expression in embryonic human neuroepithelium and in human neuroepithelial tumor cells. Lab Invest 66 (1992) 303–13.

Uchida, N., Buck, D. W., He, D., Reitsma, M. J., Masek, M., Phan, T. V., Tsukamoto, A. S., Gage, F. H., Weissman, I. L. Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci U S A 97 (2000) 14720–5.

Visvader, J. E., Lindeman, G. J. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 8 (2008) 755–68.

Wagers, A. J., Weissman, I. L. Plasticity of adult stem cells. Cell 116 (2004) 639–648.

Weller, M., Felsberg, J., Hartmann, C., Berger, H., Steinbach, J. P., Schramm, J., Westphal, M., Schackert, G., Simon, M., Tonn, J. C., Heese, O., Krex, D., Nikkhah, G., Pietsch, T., Wiestler, O., Reifenberger, G., von Deimling, A., Loeffler, M. Molecular predictors of progression-free and overall survival in patients with newly diagnosed glioblastoma: a prospective translational study of the german glioma network. J Clin Oncol 27 (2009) 5743–5750.

Wick, W., Hartmann, C., Engel, C., Stoffels, M., Felsberg, J., Stockhammer, F., Sabel, M. C., Koeppen, S., Ketter, R., Meyermann, R., Rapp, M., Meisner, C., Kortmann, R. D., Pietsch, T., Wiestler, O. D., Ernemann, U., Bamberg, M., Reifenberger, G., von Deimling, A., Weller, M. Noa-04 randomized phase iii trial of sequential radiochemotherapy of anaplastic glioma with procarbazine, lomustine, and vincristine or temozolomide. J Clin Oncol 27 (2009) 5874–5880.

Woodbury, D., Schwarz, E. J., Prockop, D. J., Black, I. B. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61 (2000) 364–370.

Yin, A. H., Miraglia, S., Zanjani, E. D., Almeida-Porada, G., Ogawa, M., Leary, A. G., Olweus, J., Kearney, J., Buck, D. W. Ac133, a novel marker for human hematopoietic stem and progenitor cells. Blood 90 (1997) 5002–12.

Yuan, H., Corbi, N., Basilico, C., Dailey, L. Developmental-specific activity of the fgf-4 enhancer requires the synergistic action of sox2 and oct-3. Genes Dev 9 (1995) 2635–45.

Zechel, C. Requirement of retinoic acid receptor isotypes alpha, beta, and gamma during the initial steps of neural differentiation of pcc7 cells. Mol Endocrinol 19 (2005) 1629–45.

Zeng, Y., Yang, Z., Xu, J. G., Yang, M. S., Zeng, Z. X., You, C. Differentially expressed genes from the glioblastoma cell line shg-44 treated with all-trans retinoic acid in vitro. J Clin Neurosci 16 (2009) 285–94.

Zhao, D., Najbauer, J., Garcia, E., Metz, M. Z., Gutova, M., Glackin, C. A., Kim, S. U., Aboody, K. S. Neural stem cell tropism to glioma: critical role of tumor hypoxia. Mol Cancer Res 6 (2008) 1819–29.

Zile, M. H. Function of vitamin a in vertebrate embryonic development. J Nutr 131 (2001) 705–8.

A Publikationen

Rasper, M., Schäfer, A., Piontek, G., Teufel, J., Brockhoff, G., Ringel, F., Heindl, S., Zimmer, C., Schlegel, J. Aldehyde dehydrogenase-1 positive glioblastoma cells show brain tumor stem cell capacity. Neuro Oncol. 12 (2010) 1024-33.

B Danksagung

Zunächst möchte ich mich ganz herzlich bei Herrn Prof. Schlegel bedanken, der mir die Möglichkeit eröffnet hat, in seinem Team meine Dissertation anzufertigen. Er führte mich nicht nur in die interessante Thematik der Tumorstammzellen ein und stellte hervorragende Arbeitsbedingungen zur Verfügung, sondern war auch ein stets hilfsbereiter Ansprechpartner während der gesamten Zeit meiner Arbeit in der Neuropathologie. Zudem möchte ich ihm für die angenehme und immer kollegiale Arbeitsatmosphäre danken, in welcher ich meine Dissertation anfertigen konnte.

Weiterhin gilt mein Dank auch Andrea Schäfer, Guido Piontek, Julian Teufel und Ingrid Hoepner, die mich allesamt nach Kräften und jeder in unersetzlicher Weise unterstützt haben. Ohne ihre Mithilfe wäre die Arbeit in ihrer jetzigen Form kaum möglich gewesen.

Besonderen Dank schulde ich auch Herrn Prof. Brockhoff, der mich durch die Durchführung der Durchflusszytometrien unterstützte und dabei ein immer hilfsbereiter und zuverlässiger Ansprechpartner war. Mein Dank gilt ebenso Herrn Dr. Ringel, der die Arbeit durch die Bereitstellung von Gliom-Resektaten entscheidend gefördert hat.

In großer Dankbarkeit seien auch meine Familie und meine Freunde erwähnt, die mich in der Zeit der Dissertation nach Kräften unterstützt haben.