A paired comparison between glioblastoma cancer stem cells and differentiated cells in view of proliferation, resistance to conventional therapies and tumour-initiating capabilities

Dissertation submitted in partial fulfillment of the requirements for the degree of “Doktor der Medizin” (Dr. med.) at the Medical Faculty of Ulm University

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A  Ampère
ABC  ATP-binding cassette
ABCB1  ATP-binding cassette sub-family B member 1
ABCG2  ATP-binding cassette sub-family G member 2
ABL  Abelson murine leukemia viral oncogene homolog
AKT/PKB  Protein kinase B
AML  Acute myeloid leukemia
Apaf-1  Apoptotic protease activating factor-1
APC gene  Adenomatous polyposis coli gene
ATM  Ataxia-telangiectasia-mutated
ATP  Adenosine-5’-triphosphate
BAD  BCL-2-antagonist of cell death
BAK  BCL-2-antagonist killer
BAX  BCL-2-associated X protein
BCA  Bicinchoninic acid
BCL-2  B-cell-lymphoma-2
BCL-xL  B-cell-lymphoma-extra large
BCR  B-cell receptor
bFGF  Basic fibroblast growth factor
BH  BCL-2-homology domain
BID  BCL-2-interacting domain death antagonist
BIM  BCL-2-interacting mediator of cell death
BSA  Bovine serum albumin
°C  Degree Celsius
CAC  Colitis associated cancer
Caspase  Cysteine-aspartic proteases
CD  Cluster of differentiation
cDNA  Complementary DNA
CFSE  Carboxyfluorescein succinimidyl ester
ChK  Checkpoint kinase
CML  Chronic myeloid leukemia
CNS  Central nervous system
Cre  Causes recombination
**Abbreviations**

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<td>CSCs</td>
<td>Cancer stem cells</td>
</tr>
<tr>
<td>DiI</td>
<td>Di-alkyl indocarbocyanine</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-inducing signalling complex</td>
</tr>
<tr>
<td>DMEM/F-12</td>
<td>Dulbecco’s modified eagle medium: nutrient mixture F-12</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DNA-PK</td>
<td>DNA-dependent-protein kinase</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
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<tr>
<td>ds</td>
<td>Differentiated glioblastoma cells</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemoluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>e.g.</td>
<td>Exempli gratia</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGFRvIII</td>
<td>EGFR variant 3</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>ESA</td>
<td>Erythropoiesis stimulating agent</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>FADD</td>
<td>Fas-associated protein with death domain</td>
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<td>Fetal calf serum</td>
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<td>Human fibroblast growth factor-2</td>
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<td>Glioblastoma</td>
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<td>g</td>
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</tr>
<tr>
<td>G1 phase</td>
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</tr>
<tr>
<td>G2 phase</td>
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<td>GAPDH</td>
<td>Glycerinaldehyde 3-phosphate dehydrogenase</td>
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<td>GCSCs</td>
<td>Glioblastoma cancer stem cells</td>
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<td>Gds</td>
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<td>GF</td>
<td>Growth factor</td>
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<td>Description</td>
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</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>gy</td>
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<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>H2AX</td>
<td>Histone 2AX</td>
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<td>H2O</td>
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<tr>
<td>HB-EGF</td>
<td>Heparin-binding EGF-like growth factor</td>
</tr>
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<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrogen chloride</td>
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<td>H.E.</td>
<td>Hematoxylin and eosin</td>
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<td>hEGF</td>
<td>Human epidermal growth factor</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>Hairy and enhancer of split1</td>
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<td>Human fibroblast growth factor</td>
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<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>H-Ras</td>
<td>Harvey rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>i.e.</td>
<td>In example</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>Il-2 rγ</td>
<td>Interleukin-2 receptor subunit gamma</td>
</tr>
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</tr>
<tr>
<td>IMS</td>
<td>Intermembrane space</td>
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<tr>
<td>IR</td>
<td>Ionising radiation</td>
</tr>
<tr>
<td>L1CAM</td>
<td>L1 cell adhesion molecule</td>
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<tr>
<td>LAMC2</td>
<td>Laminin subunit gamma-2</td>
</tr>
<tr>
<td>LOH10</td>
<td>Loss of heterogeneity of chromosome 10</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
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<td>MCL-1</td>
<td>Myeloid cell leukemia sequence-1</td>
</tr>
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<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MCSC</td>
<td>Migrating cancer stem cell</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal epithelial transition</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MGMT</td>
<td>O⁶-methylguanine-DNA methyltransferase</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
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<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair</td>
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<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilisation</td>
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<td>M phase</td>
<td>Mitotic phase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NF</td>
<td>Neurofibromatosis</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NOD-SCID</td>
<td>Non-obese diabetic-severe combined immunodeficiency</td>
</tr>
<tr>
<td>Noxa</td>
<td>Latin for damage</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural stem cell</td>
</tr>
<tr>
<td>NTC</td>
<td>Non-tumorigenic cell</td>
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<tr>
<td>Oct-4</td>
<td>Octamer-binding transcription factor-4</td>
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<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>p53</td>
<td>Protein 53</td>
</tr>
<tr>
<td>pAKT</td>
<td>Phospho-AKT</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDK1</td>
<td>3-phosphoinositide-dependent protein kinase-1</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression-free survival</td>
</tr>
<tr>
<td>pH2AX</td>
<td>Phosphorylated form of histone H2AX</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositol-3-kinase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol-3,4-bisphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------------------------------</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Phosphatidylinositol-3,4,5-triphosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>pO₂</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>pRB</td>
<td>Phosphorylated retinoblastoma protein</td>
</tr>
<tr>
<td>pS6</td>
<td>Phospho-S6</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>Rad 17</td>
<td>Radiation induced protein 17</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription-PCR</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>S6</td>
<td>S6 ribosomal protein</td>
</tr>
<tr>
<td>S6K</td>
<td>S6 ribosomal protein kinase</td>
</tr>
<tr>
<td>SC</td>
<td>Stem cell</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>Ser473</td>
<td>Serine 473</td>
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<tr>
<td>shRNA</td>
<td>Short hairpin ribonucleic acid</td>
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<tr>
<td>SOX2</td>
<td>Sex determining region Y-box 2</td>
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<tr>
<td>S phase</td>
<td>Synthesis phase</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian vacuolating virus 40</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris base, acetic acid, EDTA</td>
</tr>
<tr>
<td>tBID</td>
<td>Truncated BID</td>
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<tr>
<td>TEMED</td>
<td>Tetramethylenediamine</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor-α</td>
</tr>
<tr>
<td>Thr308</td>
<td>Threonine 308</td>
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<tr>
<td>TiCs</td>
<td>Tumour-initiating cells</td>
</tr>
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### Abbreviations

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>TM</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TMRM</td>
<td>Tetramethylrhodamine methyl ester perchlorate</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>Tris</td>
<td>Trishydroxymethyl-aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-type MMTV (mouse mammary tumour virus) integration site</td>
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</table>
1. Introduction

1.1. Cancer

In defiance of intensive scientific endeavours, cancer comes second place in the list of causes of death in the US with about 585,720 Americans expected to die of cancer in 2014, almost 1,600 people per day, surpassed only by heart disease (American Cancer Society. Cancer Facts & Figures 2014). Contemplated globally, cancers account for approximately 13% of all death cases, whereof breast cancer represents the most frequently diagnosed cancer and the leading cause of cancer death among females worldwide making up 23% of total new cancer cases and 14% of total cancer deaths in 2008. Compared with this, cancer statistics of males are headed by lung cancer accounting for 17% of total new cancer cases and 23% of total cancer deaths. Although cancer incidence rates in the developing world comprise only about half of those ascertained in economically developed countries, compared overall cancer mortalities seem to assimilate. This empirical observation is ascribed to the conjuncture that the lower incidence rate of cancer faces a poorer cancer survival rate in the developing world, most likely because of a late stage of diagnose and limited availability of standard treatment (Jemal et al. 2011).

Although risk factors for developing cancer can be apportioned to both an internal group that comprises inherited mutations, hormones and immune conditions among others and environmental and lifestyle factors, such as cigarette smoking, diet, sun exposure and environmental pollutants, these causal factors may also act together or in sequence to initiate or promote tumour formation. However, external factors are reported to be the crucial trigger for about 90-95% of all cancer cases (Anand et al. 2008, American Cancer Society. Cancer Facts & Figures 2013).

The term cancer generally applies to malignant tumours, thus implying uncontrolled division and growth of tumour cells as well as the capability to invade and destroy adjacent structures and to disseminate to sites distant from the primary lesion, whereby not all cancers feature the latter metastasising token (Kumar and Stricker 2007). Generally, cancers are classified according to the type of cell, from which they originate. On this basis tumours emerging for example in mesenchymal tissue or its derivatives are referred to as sarcomas, those derived from epithelial cells are called carcinomas, while leukemias and neuroectodermal cancers arise from hematopoietic cells and components of the central or peripheral nervous system, respectively.
Nascency of a malignant tumour cell is known to be the result of a multistep progress of sequential alterations in several genes (Croce 2008). Nevertheless, two main categories of genes can be found, which are involved in this process called carcinogenesis (Spandidos 2007). In general terms, protooncogenes stimulate cell growth and differentiation, whereas tumour suppressor genes counteract these dynamics.

Oncogenes are protooncogenes that are altered by mutations leading to constitutive activation of their resultant products, oncoproteins. Several mechanisms have been described, such as point mutations, gene fusion, juxtaposition to enhancer elements or amplification (Croce 2008). One of the most infamous examples of this concept is the ‘Philadelphia Chromosome’ in chronic myeloid leukemia (CML) cells that is initiated by a reciprocal t(9;22) chromosomal translocation, which fuses the ABL protooncogene to the BCR gene (Sudoyo and Hardi 2011). The fusion gene encodes an oncogenic ABL fusion protein with enhanced tyrosine kinase activity compared to that of the initial ABL tyrosine kinase resulting in enhanced myeloid proliferation.

As hinted above, a cancerous cell improbably rises against the backdrop of an alteration in a single oncogene, instead further mutations of tumour suppressor genes are needed. Along these lines the p53 protein has proven to be a tumour suppressor, when its activation was investigated in response to DNA damage or other forms of cellular stresses, such as hypoxia, leading to cell cycle arrest in order to allow either DNA damage to be repaired (Perkins and Gilmore 2006) or cell death through the induction of proapoptotic genes (May and May 1999). Thus, p53 is of great significance in maintaining genetic stability. Indeed, tumour suppressor genes are frequently mutated in different types of human cancers. In case of the p53 protein, it can be found a defect of its gene in more than 70 % of all human cancerous diseases, while the remaining malignant neoplasms display alterations up-stream or down-stream of p53 (Kumar and Stricker 2007).

Despite rapidly growing knowledge in cancer research, the pivotal therapeutic categories cancer is faced with have remained rather the same over the last century, as they still encompass surgery, chemo- and radiation therapy, whereby mono- or combination therapy is applied in practice depending on the location, the type and the grade of the tumour as well as the patient’s physical condition. Surgery that is geared towards removing the entire tumour mass is mainly considered promising for isolated solid cancerous lesions that lack metastases and are clearly distinguishable from the unimpaired surrounding tissue. Compared with this, chemo- and radiotherapy force malignant cells to commit suicide - also called apoptosis - by activating cell death pathways, which, as a systemic therapy, can ad-
mittedly affect metastases, but anon is limited in its administration dose due to inadvertent toxicity to other tissues.

**Apoptosis**

Although other types of programmed cell death have also been described (Sperandio et al. 2000, Debnath et al. 2005), the genetically encoded process of apoptosis (Tait and Green 2010) is the most heeded and investigated variant in medical research. In normal physiology apoptosis is decisively involved in many cellular processes as exempli gratia the initial overproduction of cells during nervous and immune system development is counteracted with apoptosis of those cells that fail to express functional synaptic connections or productive antigen specificities. As its central importance in maintaining homeostasis is manifested by the amount of about ten billion cells that undergo apoptosis every day in order to keep balance with the number of new cells arising from the stem cell pool in adult human bodies (Renehan et al. 2001), it appears that intermitting strictly regulated apoptotic pathways can give rise to pathology, such as developmental defects, neurodegenerative diseases as well as cancer.

In vertebrate cells apoptosis proceeds mainly through two pathways termed extrinsic and intrinsic, which both converge on the same signalling cascade initiated by activation of the executioner caspases-3 and -7 (Tait and Green 2010) (Figure 1). In the extrinsic pathway ligation of death receptors with their cognate ligands allows binding of adaptor proteins, such as Fas-associated death domain protein (FADD) (Wajant 2002), resulting in autocatalytic activation of procaspase-8 to caspase-8 after the trimerised receptor-ligand complex DISC has been formed (Kischkel et al. 1995). Once caspase-8 is activated, the execution phase of apoptosis is triggered, where the effector caspases-3, -6 and -7 activate cytoplasmatic endonucleases and proteases that degrade cytoskeletal proteins inter alia (Elmore 2007). By way of contrast, stress-inducing, nonreceptor-mediating stimuli, such as DNA-damage or endoplasmatic reticulum (ER) stresses (Tait and Green 2010), induce mitochondrial outer membrane permeabilisation (MOMP) that is strictly governed by the BCL-2 family of proteins, ensued by the release of proteins, such as cytochrome c, from the mitochondrial intermembrane space (Chipuk et al. 2010). Once in the cytosol, cytochrome c binds to and activates apoptotic protease-activating factor-1 (Apaf-1) resulting in the formation of the apoptosome (Chinnaiyan 1999). After recruitment to this complex procaspase-9 is converted into initiator caspase-9, which then activates executioner caspases-3 and -7 (MacFarlane 2004).
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Figure 1: The two major apoptotic pathways in mammalian cells

Although both the extrinsic A) and the intrinsic B) apoptotic pathway converge in effector caspase-3 activation resulting in the cleavage of a number of cellular substrates associated with the morphological correlate of apoptosis, the former is induced by triggering death receptors at the cell surface, whereas the latter gets activated in response to diverse stimuli that provoke cellular stress. The intrinsic pathway is strictly regulated by the BCL-2 family of proteins.

Apaf-1, Apoptosome-associated factor-1; BAD, BCL-2 antagonist of cell death; BAK, BCL-2-antagonist killer; BAX, BCL-2-associated X protein; BCL-2, B-cell-lymphoma-2; BCL-xL, B-cell-lymphoma-extra large; BID, BCL-2-interacting domain death agonist; BIM, BCL-2-interacting mediator of cell death; FADD, Fas-associating death domain protein; MCL-1, myeloid cell leukaemia sequence-1; MOMP, mitochondrial outer membrane permeabilisation; Noxa, Latin for damage; PUMA, p53 upregulated modulator of apoptosis; tBID, truncated BID.
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Apoptosis regulation via BCL-2 family members

Commitment to the intrinsic apoptotic pathway is strictly governed by members of the BCL-2 family of proteins via controlling mitochondrial permeability (Cory and Adams 2002) (Figure 1). The BCL-2 (B-cell-lymphoma-2) gene has been discovered in B-cell follicular lymphomas, where the t(14;18) translocation had coupled the immunoglobulin heavy chain locus to a chromosome 18 gene, termed BCL-2. By promoting cell survival, which is controlled separately from cell proliferation (Vaux 1988), BCL-2 spawned the concept of apoptosis inhibition to be a central step in tumour development (Cory and Adams 2002).

BCL-2 and its relatives have classically been grouped into two classes (Figure 2). The balance between the pro- and antiapoptotic repertoire is considered the main criterion for a cell to survive or commit cell death (Adams and Cory 2007) connoting that shifting the ratio towards the antiapoptotic proteins correlates to tumour cell survival and apoptosis resistance in various cancers (Adams and Cory 2007).

Pivotal to the initiation of apoptosis is activation of the effector proteins BAX and BAK that facilitate caspase activation by their effects on the outer mitochondrial membrane (OMM), where they are likely to promote MOMP by homo-oligomerisation into proteolipid pores resulting in cytochrome-c release as well as further protein diffusion from the intermembrane space into the cytosol (Chipuk et al. 2010). Being predominantly localised as a monomer in the cytosol, BAX shifts towards the OMM in order to insert as an integral membrane protein during apoptosis induction (Youle and Strasser 2008), while BAK is constitutively targeted to the OMM. However, as direct activation of BAX and BAK requires a subset of ensuing conformational changes, the mere mitochondrial localisation of these proteins does not imply that effectors are active once the transmembrane regions are embedded in the OMM (Chipuk et al. 2010). BH3-only proteins seem to induce apoptosis especially by inhibiting antiapoptotic BCL-2 family proteins so that subsequent relief of BAX and BAK concludes with MOMP and caspase activation. However, the definite cellular mechanisms involved in these activating processes are far from being grasped in their entirety (Youle and Strasser 2008). Furthermore, cytosolic BID has been shown to connect the extrinsic and intrinsic apoptotic pathway, as after caspase-8 mediated proteolysis the resulting active degradation product tBID (truncated BID) translocates to the mitochondria (Li et al. 1998) and activates BAX with subsequent caspase-9 activation to membrane permeabilisation (Lovell et al. 2008) (Figure 1).
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Figure 2: Schematic representation of the mammalian BCL-2 family of proteins
BCL-2-related proteins have classically been arranged in a pro-survival and a pro-apoptotic sub-group, whereas the former is comprised of proteins, such as BCL-2 itself, BCL-xL and MCL-1 that exhibit two or four conserved BCL-2-homology (BH) domains and are typically integrated within the OMM in order to protect the mitochondria against MOMP (Figure 1), but can also be found in the cytosol or ER membrane. The proapoptotic BCL-2 repertoire is divided again into the downstream apoptosis effector proteins BAK and BAX that possess BH1, BH2, BH3 (Lindsten et al. 2000, Cheng et al. 2001) as well as family members that comprise the BH3 domain only, therefore denoted BH3-only proteins. ‘Sensitiser’ or ‘derepressor’ BH3-only proteins, such as BAD, PUMA and Noxa, fulfill their functions by linking to the antiapoptotic BCL-2 proteins, whereas BID and BIM as ‘direct activators’ are suggested to additionally interact with the effector proteins BAK and BAX (Chipuk et al. 2010).

BAD, BCL-2 antagonist of cell death; BAK, BCL-2-antagonist killer; BAX, BCL-2-associated X protein; BCL-2, B-cell-lymphoma-2; BCL-xL, B-cell-lymphoma-extra large; BH, BCL-2-homology domain; BID, BCL-2-interacting domain death agonist; BIM, BCL-2-interacting mediator of cell death; MCL-1, myeloid cell leukemia sequence-1; MOMP, mitochondrial outer membrane permeabilisation; Noxa, Latin for damage; OMM, outer mitochondrial membrane; PUMA, p53 upregulated modulator of apoptosis; TM, transmembrane domain. Scheme modified from Strasser 2005 with permission from Nature Reviews Immunology.
1. Introduction

The PI3K/AKT signalling

The phosphatidylinositol-3-kinase/AKT (PI3K/AKT) pathway is one of the most highly mutated signalings in human cancer (Engelman et al. 2006). Exhibiting exceeding activation, it results in disorder of control of cell growth and survival and contributes to a competitive growth advantage, metastatic ability and therapy resistance (Hennessy et al. 2005). The generation of phosphatidylinositol-3,4,5-trisphosphate (PIP$_3$) out of phosphatidylinositol-3,4-bisphosphate (PIP$_2$) through phosphatidylinositol-3-kinase (PI3K) that has been stimulated by activated receptors recruits AKT - also known as protein kinase B (PKB) - to PIP$_3$ at the plasma membrane. A serine/threonine kinase named 3-phosphoinositide-dependent protein kinase-1 (PDK1) subsequently phosphorylates AKT on Thr308 leading to partial AKT activation. However, maximal activation requires subsequent phosphorylation of Ser473 (Vivanco and Sawyer 2002), which is thought to be mediated by several kinases, such as mTOR complex 2 (Sarbassov et al. 2005) and DNA-dependent protein kinase (DNA-PK) (Feng et al. 2004). Among the plethora of substrates of activated PKB, the serine/threonine kinase mTOR complex 1 carries great weight, as it regulates translation in response to growth factors by phosphorylating components of the protein synthesis machinery, such as S6-kinase (S6K), which phosphorylates the ribosomal protein S6, and thereby promoting protein synthesis and cell proliferation (Hennessy et al. 2005) (Figure 3).

Several modes by which AKT inhibits cell death have been identified so far. Among them, direct interactions with proapoptotic BCL-2 family proteins are of particular interest. On this view AKT has shown to be able to phosphorylate BAD resulting in cytosolic sequestration and inactivation of BAD and reduced urge to bind and antagonise the proapoptotic protein BCL-xL (Del Peso et al. 1997). In addition, AKT-mediated phosphorylation of BAX has been reported to inhibit conformational change of this proapoptotic BCL-2 family member as well as its subsequent translocation to mitochondria, thus promoting disruption of mitochondrial membrane potential, cytochrome-c release, caspase activation and apoptosis (Yamaguchi and Wang 2001). BIM and caspase-9 have been demonstrated to feature other phosphorylation sites of AKT resulting in protein inactivation (Qi et al. 2006, Cardone et al. 1998) (Figure 3).
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Figure 3: Overview of the PI3K/AKT/mTOR signalling

Binding of growth factors (GF) to receptor tyrosine kinases (RTK) results in recruitment and activation of PI3K. Subsequently, phosphorylation of PIP$_2$ to PIP$_3$ initiates the activation of AKT by PDK1 and mTOR complex 2. Signallings of activated AKT are implicated in promoting a plethora of cellular processes including cell survival and protein synthesis.

BAD, BCL-2 antagonist of cell death; BAX, BCL-2-associated X protein; BIM, BCL-2-interacting mediator of cell death; GF, growth factor; MOMP, mitochondrial outer membrane permeabilization; mTOR, mammalian target of rapamycin; PDK1, 3-phosphoinositide-dependent protein kinase-1; PIP$_2$, phosphatidylinositol (3,4)-bisphosphate; PIP$_3$, phosphatidylinositol (3,4,5)-trisphosphate; PI3K, PI3 kinase; PTEN, phosphatase and tensin homolog; p70S6K, p70S6 kinase; RTK, receptor tyrosine kinase; S6, S6 ribosomal protein.
1.2. **Cancer stem cells**

The concept of cancer stem cells (CSCs) that was first introduced by Lapidot et al. almost 20 years ago proposes a subpopulation of highly malignant cancerous cells to represent the top of a tumour cell hierarchy comparable to hierarchically organised adult regenerating tissues, where normal adult stem cells preserve tissue regeneration (Valent et al. 2012). Back then, Lapidot and colleagues identified AML-initiating cells by transplanting human acute myeloid leukemia cells into severe combined immunodeficient (SCID) mice resulting in a heterogenous leukemic cell population, which showed morphological and dissemination features similar to that of the donor’s disease. In contrast to other tumour cells, these cells were denoted by expression of the cell-surface markers CD34⁺ CD38⁻ (Lapidot et al. 1994). As few as 5000 CD34⁺ CD38⁻ cells have proven to be sufficient to initiate tumour growth in mice, whereas 500,000 CD34⁺ CD38⁺ cells of the residual tumour cell population were not able to engraft. To determine whether CD34⁺ CD38⁻ tumour initiating cells that represented less than 0.2 % of the total leukemic cells exhibited the capacity to self-renew - that is to say the ability to give rise to a new tumour initiating cell after cell division - leukemic cells now taken from the tumour in mice were transplanted serially into secondary recipients. Again, only the newly isolated CD34⁺ CD38⁻ cells were capable of self-renewal and differentiation (Bonnet and Dick 1997). Up to now, the only universally agreed upon defining feature of CSCs is their ability to initiate tumours in immunosuppressed animals that even after serial transplantation (imperfectly) recapitulate the malignancy found in the patient (Baccelly and Trumpp 2012). These CSCs are capable of both expansion of the cancer stem cell pool and differentiation into the heterogenic cancer cells that represent the tumour bulk (Clarke et al. 2006). Considering their ability to generate the donor’s tumour in mice again, CSCs are often termed tumour-initiating cells (TiCs) synonymously (Cruceru et al. 2013, Zhou et al. 2009).

In 2003, CSCs were first isolated from tumours of the breast as solid tumours (Al-Hajj et al. 2003). So far, hierarchical organisation of tumours headed by TiCs has been reported for several other cancers, such as colon cancer (Ricci-Vitiani et al. 2007), prostate cancer (Collins et al. 2005), brain cancer (Singh et al. 2004) as well as many others.

In contrast to the idea of a hierarchical organisation of many tumours, findings of Odoux and colleagues that suggest marked chromosomal instability to be present in colon cancer stem cells support a stochastic model of cancer in the cancer stem cell population, where genetic instability leads to the formation of new genetic varied CSC clones despite of
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originating from the same initial CSC clone. Together with the biologic selection of CSCs that prove to be best adapted for survival, proliferation, invasion and metastasis, heterogeneous stem cell populations may be generated within a single tumour and may offer one possible explanation for treatment failure (Odoux et al. 2008).

The attempt to bring in line both of these ostensible competing models of tumour biology spawns a hierarchical mode of tumour growth that at the cellular level is mediated by stochastic cell fate decisions (Driessens et al. 2012).

While the role of CSCs in tumour genesis is still disputed, the potential origins of these cells are also far from clear. Thus, CSCs could originate from stem cells that have been malignant transformed by accumulation of oncogenic insults over time. For instance, using model organisms, Barker and colleagues were able to transform intestinal stem cells localised as crypt bottoms by deletion of APC in these cells. Macroscopic adenomas were obtained within three to five weeks (Barker et al. 2009). By way of contrast, TiCs could also arise from a more differentiated progenitor that has acquired stem cell properties by a subset of genetic or epigenetic abnormalities (Dean et al. 2005). There is even evidence that cells with CSC properties emerge during transformation of totally differentiated cells. On this view primary skin fibroblasts were transformed in vitro by expression of telomerase (hTERT), the oncogenic H-RasV12 mutant and concomitant inhibition of p53 and pRB (phosphorylated retinoblastoma protein) pathways by simian virus 40 (SV40) Large-T and Small-T antigens. The subset of transformed fibroblasts was reprogrammed to a more primitive, multipotent cell type that presented many hallmarks of CSCs and was able to give rise to hierarchically-organised tumours (Scaffidi and Misteli 2011).

Whatever the process of generating CSCs might be, it increasingly becomes apparent that CSCs in this context may not be mistaken as cells of origin regarding the respective malignancies, as they may arise out from other cells after transformation.

The CSC concept is of key importance clinically, as it may explain why many treatments appear to be effective initially, but tumours reappear later (Valent et al. 2012). Against this background, TiCs have been described to potentially remain dormant (Martin-Padura et al. 2012) and to be capable to reversibly switch from dormancy to self-renewal (Wilson et al. 2008). As neoadjuvant chemo- and radiotherapy is said to mainly target rapid cycling cells, TiCs hereby could withstand conventional therapies and induce new tumour formation, although the tumour mass has been eliminated successfully. This hypothesis also builds on
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various features that might make TiCs seem more resistant to several therapeutic strategies than their differentiated descendants. In a study of Diehn et al. 2009, the expression of a number of antioxidant genes was highly upregulated in primary human breast CSCs compared to non-tumourigenic cells (NTCs) and therefore a significant lower reactive oxygen species (ROS) level could be detected in the CSC-enriched population. Taken into account that free radicals are regarded as a major fatal event for tumour cells in consequence of exposure to ionising radiation (IR), the authors could also expectably observe fewer DNA-strang breaks in the breast cancer stem cells than NTCs immediately after IR (Diehn et al. 2009). The underlying mechanisms concerning resistance to DNA damage-inducing agents can at least partly be traced to the expression of high levels of ATP-binding cassette (ABC) transporters that use the energy of ATP hydrolysis to efflux drugs from cells. ABC transporters have proven to be an important aid in the isolation and analysis of hematopoietic stem cells, as the fluorescent dye Hoechst 33342 does not accumulate in these cells because of its discharge by ABCG2 and ABCB1. Inhibitors of these proteins might be useful in killing tumour stem cells. Two agents - GF120918 and tariquidar - that inhibit both ABCG2 and ABCB1 are already approved for clinical studies.

In summary, CSCs probably exhibit many of the properties of normal stem cells including relative quiescence, resistance to drugs and active DNA-repair capacity, what makes them difficult to eliminate (Dean et al. 2005).

Because of recent evidence portending that TiCs differently respond to antitumour agents in vitro and in vivo (Valent et al. 2012), the idea of CSC niches is also a subject of debate especially regarding the responses of TiCs to a given treatment. Various different cell types are involved in generating such niche environments, including immune cells (Shiao et al. 2011), stromal cells and extracellular components (Hanahan and Coussens 2012). It is very likely that tumour microenvironment supports CSCs to exert their crucial dimension. For instance, interleukin-6 secreted by infiltrating immune cells enhances proliferation of TiCs within colitis associated cancer tumours (Grivennikov et al. 2009). Vermeulen et al. demonstrated that secreting of hepatocyte growth factor (HGF) by tumour-associated myofibroblasts could reprogram differentiated cancer cells that had lost their tumour-initiating potential to express CSC markers again and regain their tumourigenic capacity suggesting that stemness is partly orchestrated by tumour microenvironment (Vermeulen et al. 2010).

In the field of invasion and metastasis, TiCs are more and more believed to occupy a leading dimension. In order to become motile and invasive, carcinoma cells have to undergo
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Epithelial mesenchymal transition (EMT), which facilitates delaminating from the primary tumour by losing cell adhesion features, intravasation into lymph or blood vessels, extravasation at secondary sides and forming a new carcinoma through mesenchymal epithelial transition (MET) leading to epithelial redifferentiation (Thiery 2002). In colorectal cancer, stem cell-associated Wnt target genes, such as survivin, can be detected very early in carcinogenesis and remain highly expressed during tumour progression. Such cells, including differentiated and dedifferentiated variants, are located throughout all areas of carcinomas and metastases. Genes that are linked with EMT, among them L1CAM or LAMC2, are downregulated in differentiated tumour cells within the tumour bulk, but expression is mainly observed in disseminated tumour cells. Hence, colorectal CSCs can be classified into two groups: stemness/proliferation - found throughout all progression steps - and EMT/dissemination - observed mainly at the invasive front of the tumour. These observations led to the concept of migrating cancer stem cells (MCSCs), where stationary cancer stem cells face mobile cancer stem cells. By acquiring temporary EMT, MCSCs comprise the two perhaps most decisive traits, i.e. stemness and mobility (Brabletz et al. 2005).

Against this background, it seems to be reasonable that recent focus of therapeutical intervention has shifted towards targeting CSCs, in particular difficult to treat malignancies, such as glioblastoma.

1.3. Glioblastoma

Within all gliomas, glioblastoma - classified as a World Health Organization (WHO) grade IV astrocytoma - accounts for more than 50% of these tumours and therefore can be considered the most common malignant primary brain tumour (Ostrom et al. 2013, Osborn et al. 2012, Wen and Kesari 2008). Primary glioblastoma, which comprises the majority (>90%) of cases and is usually seen in older patients with a mean age of about 60 years, emerges de novo without evidence of a less malignant precursor lesion. The rapid growth of this entity entails a short clinical history of less than three months, usually. Compared to this, progression of low-grade or anaplastic astrocytoma can create secondary glioblastoma (Kesari 2011), which generally affects middle-aged individuals. Magnetic resonance imaging (MRI) typically reveals large tumours characterised by central necrosis, ring enhancement and perifocal oedema (Ohgaki 2005). Both primary and secondary glioblastomas histologically appear as necrotic, hemorrhagic and infiltrating masses revealing distinct
nuclear polymorphism, increased mitosis and pseudo-palisading of tumour cell nuclei (Frosch 2007) (Figure 4).

**Figure 4: Glioblastoma**
A) Computed tomography imaging showing irregular hypodense foci representing necrosis, intense enhancement of the margins as well as surrounding oedema.
(Special gratitude to Prof. Dr. M.-E. Halatsch for kindly providing the image, Department of Neurosurgery, Ulm University Medical Center).
B) H.E. (hematoxylin and eosin) staining revealing a densely cellular tumorous mass and pseudo-palisading of tumour cell nuclei (signed with arrows).
(Special gratitude to Prof. Dr. D. Thal for kindly providing the image, Laboratory of Neuropathology, Ulm University (© Dietmar Thal)).

Unfortunately, despite of the therapeutic modalities that range from surgical resection alone to surgery followed by radiotherapy with concurrent and adjuvant chemotherapy treatment remains palliative for most patients illustrated by a mean patient survival of about 15 months (Preusser et al. 2011). This therapeutic failure is at least in part due to the unique growth pattern of glioblastoma. While metastasis outside the brain is virtually unheard of, glioblastoma features a wide dissemination within normal brain tissue giving rise to satellite lesions even several centimeters away from the tumour bulk illustrating glioblastoma to represent a whole brain disease (Snuderl et al. 2011, Ene and Fine 2011). After the tumour has been surgical removed, it relapses in more than 95 % of cases within two to three centimetres of the resection cavity (Giese et al. 2003). Recurring tumours can only be countered with virtually powerless therapeutic options as reflected by a life expectancy of 25 weeks after disease progression has been evaluated by use of contrast-
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eenhanced computed tomography (Wong et al. 1999). Further problems, which add to poor prognosis of glioblastoma, include the lack of lipophilic chemotherapeutic agents that are able to cross the blood-brain barrier as well as the rapid development of resistance to these few substances that are on hand, which is considered a direct consequence of genetic transformation and glioblastoma heterogeneity (Stupp et al. 2007).

Research over the last three decades has revealed a defined set of oncogenic pathways being of particular importance in understanding the pathogenesis of glioblastoma. Among these altered signalings the PI3K/AKT pathway together with its negative regulator phosphatase and tensin homolog (PTEN) is believed to occupy an important role. PTEN was identified in the course of the research for tumour suppressor genes on chromosome 10, as loss of one entire copy of chromosome 10 was considered a common characteristic of glioblastoma (Bigner et al. 1988). Indeed, Loss of Heterogeneity of Chromosome 10 (LOH10) has proven to be the most frequent alteration, whereby in 60-80 % secondary glioblastoma exhibited partial or complete loss of chromosome 10q, but no loss of 10p, compared to the lack of the entire chromosome 10 in primary glioblastoma (Ohgaki et al. 2004). By encoding a phosphatidylinositol-3,4,5-triphosphate 3-phosphatase, PTEN is able to educe a tumour suppressor (Tamura et al. 1998, Lee et al. 1999). Generally spoken, catalysis of PIP
$_3$
 to PIP
$_2$
 leads to inhibition of the AKT signalling pathway and therefore decreases cell proliferation and survival. Mutation or deletion of the PTEN gene at 10q23 is a common event in primary glioblastoma occurring in about 32 % of these tumours, whereas it is rarely found in secondary glioblastoma (4 %) (Tohma et al. 1998). Furthermore, abnormal proliferation also coheres with EGFR gene amplification found in more than 40 % of primary glioblastoma, as EGFR overexpression is seen in all these cases. By recruitment of phosphatidylinositol-3-kinase (PI3K) to the cell membrane, activation of the EGFR receptor also affects the activation of the AKT pathway (Ohgaki and Kleihues 2009) and thereby attaches growing importance to this proliferation-promoting pathway, again. Despite the increasing interest in these signalling, clinical outcome has remained rather sobering.

Expression of the O$_6$-methylguanine-DNA methyltransferase (MGMT) gene located at chromosome 10q26 has also let to an explosive interest, as the resulting DNA repair protein is able to inhibit cross-linking of double-stranded DNA by removing alkyl adducts caused by alcylation agents, such as temozolomide, from the O$_6$ position of guanine (Pegg 1990). Methylation of the O$_6$ position provokes activation of the mismatch repair (MMR) system resulting in G$_2$ checkpoint activation and subsequent G$_2$/M cell cycle rest, which
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paves the way for inducing apoptosis (Stupp et al. 2007). So, high levels of MGMT activity in glioblastoma are associated with resistance to alkylating chemotherapy. However, it has been shown that MGMT, which is epigenetically silenced by promotion methylation, exhibits significantly diminished DNA-repair activity. This epigenetic inactivation is believed to be found in approximately 75% of secondary and 36% of primary glioblastoma (Ohgaki et al. 2004). In a study published by Hegi et al., patients with MGMT promoter methylation suffering from glioblastoma and treated with temozolomide (TMZ) plus radiotherapy showed a median overall survival of 22 months compared to 13 months of those without this epigenetic variant (Hegi et al. 2005). Hence, the methylation status of the MGMT promoter is assumed to be the strongest predictor for outcome and benefit to temozolomide treatment (Stupp et al. 2007).

As TMZ induces G2/M arrest and cells treated with irradiation are most vulnerable to DNA damage in this cell cycle stoppage, concomitant radiochemotherapy has become a backbone in glioblastoma treatment. Standard of care is made up of maximum surgical debulking and subsequent focal radiotherapy completed by concurrent and adjuvant administration of TMZ (Stupp et al. 2007).

Since myelosuppression observed in less than 10% of patients appears to be the most severe side effect and no increase in non-hematologic toxicities or neurocognitive deficits are reported, this standard treatment is said to be well tolerated (Stupp et al. 2007). However, in default of further chemotherapeutic agents that are capable to cross the blood brain barrier, therapy strategy remains unchanged in case of unmethylated MGMT promoter state, although prospects of success are dispiriting in these situations. Hence, further therapeutic investigation is instantly needed.

1.4. Glioblastoma cancer stem cells

Within the last decade, several studies provided supporting evidence for the existence of CSCs in glioblastoma. In 2002, Ignatova et al. identified stem-like cells in adult malignant gliomas from the cerebral cortex that were able to form neurospheres in vitro (Ignatova et al. 2002). Two years later, a CD133+ cell subpopulation that was isolated from human glioblastomas and expressed the precursor cell markers nestin and vimentin was shown to initiate tumours in non-obese diabetic–severe combined immunodeficient (NOD-SCID) mice, whereas CD133− cells failed to (Singh et al. 2004). A large part of the neurooncological community believes that targeting TiCs is essential for therapeutic improvement (Nduom et al. 2012, Persano et al. 2012). As a result, much recent research on glioblas-
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toma has shifted towards this tumour subpopulation in order to understand its role in glioblastoma genesis and recurrence and how it might be targeted specifically.

Several different mechanisms and cell types have been suggested that glioblastoma could be generated from. One possible source builds upon the knowledge in the field of neural stem cells (NSCs). According to that, glioblastoma cancer stem cells (GCSCs) might be engendered from genetic and epigenetic changes in neural stem cells and neural progenitor cells (Huang et al. 2010), whereby the latter are defined by their limited ability to only produce progeny along either neuronal or glial lineages, but not both (Sanai et al. 2005). Multipotent and self-renewing NSCs have been isolated from the subventricular zone as well as the lining of the lateral ventricles, the dentate gyrus, within the hippocampus and the subcortical white matter. The observation that many gliomas are localised within the periventricular zone or adjacent to this area between the striatum and the lateral ventricle (Sanai et al. 2005) additionally supports this notion of GCSC ancestry. Furthermore, the ability to gender GCSCs is also attributed to differentiated cells in the CNS. In a mouse model using Cre-inducible lentiviral vectors that carried out two short hairpin RNAs (shRNA) - one targeting the gene encoding neurofibromatosis type 1 mutated in 18 % of glioblastoma and the other targeting p53 mutated in over 35 % of glioblastoma - Friedmann-Morvinski et al. could show that differentiated GFAP-positive astrocytes upon these defined genetic alterations undergo de-differentiation to generate a GFAP-negative cell, which expresses nestin and can maintain pluripotency and perhaps tumour progression (Friedmann-Morvinski et al. 2012). This capacity of a differentiated glial cell to attain continual self-renewal, thus acquiring stem cell properties, could significantly concur to glioma recurrence after treatment, as any tumour cell that is not killed can continue to proliferate and induce new tumour formation.

In analogy to the important findings regarding niche environments in other cancers, discussed above, and the clearly defined locations, where neural stem cells preferentially reside, such as the subventricular zone (Reynolds et al. 1992), the site of a putative CSC-niche is also sought in terms of glioblastoma. Nestin+/CD133+ tumour cells that included the CSC fraction have been found next to capillaries in glioblastoma. Furthermore, there is strong evidence that the self-renewing and undifferentiated state of brain CSCs is sustained by endothelial-cell-secreted factors. By increasing the quantity of endothelial cells or blood vessels in orthotopic brain tumour xenografts, an increase in the number of nestin+/CD133+
positive cells as well as a faster tumour initiation and growth could be detected. Taken together, this supports the idea that brain tumours orchestrate vascular niches, in which CSCs reside, and that endothelial cells constitute a major component in forming these niche environments (Calabrese et al. 2007). By co-culturing of tumour-derived microvascular endothelial cells and GCSCs, direct interaction between these two cell types has proven to be necessary for the stimulation of proliferation and expansion on the one hand and the prevention of GCSCs differentiation on the other hand (Borovski et al. 2009). Dependent on tumour stem cell localisation, a significantly different phenotype of CD133\(^+\) GCSCs was observed. The CD133\(^+\) cells in vascular niches showed higher expression levels of stem cell markers, such as SOX2, EGFR and nestin, whereas single CD133\(^+\) cells located throughout the tumour mass proved to be more proliferating and exhibited an endothelial differentiation profile (Christensen et al. 2011). A study of Ricci-Vitiani et al. revealed that quite a number of endothelial cells in glioblastoma carried the same glioblastoma-specific chromosomal aberrations as tumour cells. The presence of human-derived endothelial cells in tumours - generated by orthotopic or subcutaneous injection of human GCSCs in immunocompromised mice - implies the capacity of CSCs to differentiate in endothelial cells and therefore directly contribute to the tumour vasculature (Ricci-Vitiani et al. 2010). It seems likely that GCSCs are essentially involved in creating their own vascular niche that may provide a specific microenvironment for the maintenance of GCSCs.

In addition, a separate body of literature also implies hypoxic regions to represent appropriate sites, where CSCs may reside (Li et al. 2009). In analogy to hematopoietic stem cells that have been found to be located in regions with very low pO\(_2\) levels (Chow et al. 2001) and observations that hypoxic conditions are necessary for maintaining pluripotency and reducing the amount of spontaneous cell differentiation of human embryonic stem cells (Ezashi et al. 2005), it was hypothesised that hypoxia may contribute to a cancer stem cell niche (Gilbertson and Rich 2007, Keith and Simon 2007). Indeed, under both normoxic and hypoxic conditions GCSCs express hypoxia inducible factor 2\(\alpha\) (HIF\(_2\)\(\alpha\)) protein at higher level than nonstem tumour cells and even normal neural stem cells. Besides, HIF\(_2\)\(\alpha\) expression has been associated with poor survival of patients suffering from glioblastoma.

In comparison, HIF\(_1\)\(\alpha\) is upregulated in both normal and cancer stem cells. These findings indicate that HIF\(_2\)\(\alpha\) could be a specific target of anti-GCSC therapies (Li et al. 2009). Taking into account that HIF\(_2\)\(\alpha\) is also expressed by GCSCs at normal tissue oxygen levels and that CSCs can be a crucial source of key angiogenic factors, such as vascular endothelial growth factor (VEGF), and therefore promote tumour angiogenesis (Bao et al. 2006), this
data set also implies that CSCs can support the development and maintenance of their own, in this case hypoxic microenvironment (Li et al. 2009).

Hypoxic and perivascular niches harbouring CSCs might offer potential therapeutic strategies based on vascular targeting (Li et al. 2009). In this context, bevacizumab, a monoclonal antibody against VEGF, was approved in 2009 by the US Food and Drug Administration as a single agent for the treatment of recurrent glioblastoma. Ever after the field of application has expanded in the clinical settings on primary glioblastoma, too (Rahmathulla et al. 2013). However, the effectiveness of bevacizumab is a subject of highly controversial debate. The published clinical studies frequently show, what appears to be quite opposite results. For example in one trial, after patients with newly diagnosed glioblastomas had been treated with radiation plus concomitant temozolomide plus bevacizumab after maximum surgical debulking with functional preservation, the median progression-free survival (PFS) and the median overall survival (OS) accounted for 13 and 23 months, respectively (Narayana et al. 2012). Therefore, this particular combination treatment appeared to be more effective than radiotherapy plus temozolomide alone, where 6.9 and 14.6 months were reported for PFS and OS (Stupp et al. 2005). In contrast, a phase III trial evaluating bevacizumab in patients with newly diagnosed glioblastoma showed OS of 16 months whether patients received chemoradiation alone or together with the angiogenesis inhibitor. Besides, differences in PFS also did not meet the trial’s definition of statistical significance (p < 0.002), whereas adverse events, such hypotension and venous thromboembolism, were associated with the use of bevacizumab (Gilbert et al. 2014).

These controversial data suggest that unknown cofactors, such as the genetic make-up of the individual tumours treated, might contribute to the success or failure of this therapeutic antibody. In my assessment one should be highly cautious of using bevacizumab in a clinical setting, while these possible co-factors as well as the biological and clinical side effects of chronic VEGF inhibition are not fully elucidated.
1.5. **Aim of the study**

Against the backdrop of current scientific knowledge regarding cancer stem cells (CSCs) we examined the hypothesis that several frequently postulated key features of CSCs were also valid for the pathology of glioblastoma cancer stem cells (GCSCs). Utilising three matched pairs of glioblastoma cell populations, we elucidated potential differences between GCSCs and their differentiated progeny with regard to proliferation and conjectural resistance to standard treatment. Furthermore, protein expression of both, differentiation and stem cell markers, as well of members said to be crucial for glioma signalling cascades were examined.

Finally, by applying an orthotopic animal model, one matched pair was xenotransplanted into the brain of immunocompromised mice and resulting tumours were analysed.
2. Materials and Methods

2.1. Material

Chemicals were purchased from Sigma Aldrich, except when stated otherwise.

2.1.1. Cell lines

<table>
<thead>
<tr>
<th>G35 TiC</th>
<th>Primary tumour material</th>
</tr>
</thead>
<tbody>
<tr>
<td>G35 d</td>
<td>Primary tumour material</td>
</tr>
<tr>
<td>G38 TiC</td>
<td>Primary tumour material</td>
</tr>
<tr>
<td>G38 d</td>
<td>Primary tumour material</td>
</tr>
<tr>
<td>G40 TiC</td>
<td>Primary tumour material</td>
</tr>
<tr>
<td>G40 d</td>
<td>Primary tumour material</td>
</tr>
</tbody>
</table>

Tumour-initiating cells (TiCs) were isolated from patient tumour material provided by University Medical Center Ulm, Department of Neurosurgery, Günzburg, Germany. Subsequently, differentiated progeny (ds) were generated as described in the methodical part.

2.1.2. Cell culture reagents

- Dulbecco’s modified eagle medium (DMEM) Life Technologies, Inc.
- Dulbecco’s modified eagle medium: nutrient mixture F-12 (DMEM/F-12) Life Technologies, Inc.
- Fetal Calf Serum Biochrom
- Penicillin/Streptomycin Invitrogen
- L-Glutamine Invitrogen
### 2. Materials and Methods

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungizone</td>
<td>Biochrom</td>
</tr>
<tr>
<td>B27 supplement without Vitamin A</td>
<td>Life Technologies, Inc.</td>
</tr>
<tr>
<td>Human basic fibroblast growth factor 2 (Human bFGF-2)</td>
<td>Miltenyi Biotec Inc.</td>
</tr>
<tr>
<td>Human epidermal growth factor (Human EGF)</td>
<td>Biomol</td>
</tr>
<tr>
<td>HEPES buffer</td>
<td>Biochrom</td>
</tr>
<tr>
<td>Trypsin/EDTA solution</td>
<td>Biochrom</td>
</tr>
<tr>
<td>TrypsinLE Express</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Trypan blue</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

#### 2.1.3. Chemotherapeutic agents

- Temozolomide (100 mM) provided by the pharmacy of Children’s Hospital Ulm

#### 2.1.4. FACS analysis

- Propidiumiodide: Sigma
- LysotrackerRed: Invitrogen
- Tetramethylrhodamine methyl ester perchlorate (TMRM): Sigma

#### 2.1.5. Protein electrophoresis and Western blot analysis

- Polyacrylamid Rotiphorese 30: Merck KGaA
- TEMED: AppliChem
- Protease inhibitor cocktail: Roche Diagnostics
- Ammoniumpersulfate: Serva
- Sodium fluoride: Sigma
- Dithiothreitol (DTT): Sigma
- Dithiothreitol (DTT): Sigma
- Sodiumorthovanadat: Sigma
- 2-Mercaptoethanol: Merck KGaA
- B-Glycerophosphate: Sigma
- Bovine Serum Albumin: Serva
2. Materials and Methods

Bicinchoninic acid (BCA) Protein Assay Reagent Kit  
Thermo scientific

Protein Marker Page Ruler  
Thermo scientific

Milk powder  
Roth

Hybond ECL Nitrocellulose membrane  
Amersham Bioscience

Enhanced Chemiluminescence  
Amersham Bioscience

Hyperfilm ECL  
Bioscience

2.1.6. Antibodies

Western blot primary antibodies

- rabbit anti-BAX polyclonal antibody 1:500  Upstate
- rabbit anti-BAK polyclonal antibody 1:1000  BD Pharmingen
- rabbit anti-BAD polyclonal antibody 1:1000  Cell Signaling
- rabbit anti-BID polyclonal antibody 1:1000  Cell Signaling
- mouse anti-PUMA/bbc3, N-Terminal 1:500  Sigma
- mouse anti-BCL-2 monoclonal antibody 1:1000  BD Pharmingen
- rabbit anti-BCL-xL monoclonal antibody 1:1000  BD Bioscience
- rabbit anti-MCL-1 monoclonal antibody 1:1000  Cell Signaling
- rabbit anti-phospho-AKT (Ser473) polyclonal antibody 1:1000  Cell Signaling
- rabbit anti-phospho-AKT (Thr308) polyclonal antibody 1:1000  Cell Signaling
- mouse anti-AKT monoclonal antibody 1:500  BD Bioscience
- rabbit anti-phospho-S6 ribosomal protein (Ser235/236) polyclonal antibody 1:1000  Cell Signaling
- rabbit anti-S6 ribosomal protein polyclonal antibody 1:1000  Cell Signaling
- rabbit anti-caspase-3 polyclonal antibody 1:1000  Cell Signaling
- mouse anti-phospho-ERK (Thr202/Tyr204) monoclonal antibody 1:2000  Cell Signaling
- rabbit anti-ERK polyclonal antibody 1:10000  Cell Signaling
- mouse anti-α-tubulin monoclonal antibody 1:5000  Calbiochem
- mouse anti-β-actin monoclonal antibody 1:10000  Sigma
- mouse anti-nestin monoclonal antibody 1:1000  Cell Signaling
## 2. Materials and Methods

### Western blot secondary antibodies
- goat anti-mouse IgG conjugated to HRP 1:5000 Santa Cruz Biotechnology
- goat anti-rabbit IgG conjugated to HRP 1:5000 Santa Cruz Biotechnology

### Fluorescence microscopy primary antibodies
- rabbit anti-BAD polyclonal antibody 1:100 Cell Signaling
- rabbit anti-BID polyclonal antibody 1:100 Cell Signaling
- rabbit anti-MCL-1 monoclonal antibody 1:100 Cell Signaling

### Fluorescence microscopy secondary antibodies
- Goat anti-rabbit IgG antibody, FITC conjugate 1:200 Millipore

### 2.1.7. RT-PCR and related material

#### RNA Isolation, Reverse transcription and PCR amplification
- RNeasy Mini Kit Qiagen
- PCR-Master Mix Promega
- 6 xL-Loading Dye Solution Fermentas
- 100 bp DNA ladder Invitrogen
- ImPro-II Reverse Transcription System Promega
- Agarose Sigma
- Ethidium bromide Merck KGaA

#### PCR-Primers
- BID Biomers
  - forward: 5’-ACA AAT ACG AAT GTG CAG CG-3’
  - reverse: 5’-CAG CTC CGA CTC ATT CCT G-3’
- Noxa Biomers
  - forward: 5’-AGC AGA GCT GGA AGT CGA GTG TG-3’
  - reverse: 5’-TGA TGC AGT CAG GTT CCT GAG C-3’
2. Materials and Methods

GAPDH
forward: 5’-GAA GGT GAA GGG AGT-3’
reverse: 5’-GAA GAT GGT GGA TTT-3’

2.1.8. Further material

Casyton dilution liquid
Dimethyl sulfoxide (DMSO)
Mounting medium for fluorescence
Phosphate buffered saline (PBS)
MitoTracker-Red CMXRos
CFSE
PBS

2.1.9. Plasticware

All plastic material was supplied by BD Falcon, except:

- Pipettes Costar Stripette 5-25ml
- Cryotube 1.5ml
- Combitipps
- Safe-lock tubes

2.1.10. Hardware

- Agarose Gel Apparatur
- Centrifuge 5417C
- Centrifuge Varifuge 3.0 R
- Centrifuge Biofuge Fresco
- Optimax Developer
- Mastercycler gradient
- MS Minishaker
- Shaker Polymax 2040
- Thermomixer Comfort
2. Materials and Methods

Electrophoresis Power Supply EPS 300 Biotech
FACS Scan FACSCalibur Beckton Dickinson
LSR II Flow Cytometer BD Biosciences
Incubator BBD 6220 Heraeus Instruments
Pipetboy acu IBS Integra Biosciences
Multipipette Plus Eppendorf Biosciences
Pipettes 1 - 1000µl Gilson
Transblot SD BioRad Laboratories

Microscope AX 70 ‘Provis’ Olympus
Microscope CK 30 Olympus
Vortex Genie 2 Scientific Industries

Waterbath Kötttermann-Rowa
Cell counter Casy®1 Schärfe System
ELISA ELx 800 BioTek Instruments Inc

Gel Imager Intas UV-Systeme Intas
TransBlot SD BioRad Laboratories

Ultrospec 2000 UV/Visible Photometer Pharmacia Biotech
Scanner hp Scanjet G4050 Hp
Cell Strainers BD Falcon

2.1.11. Software

CFSE-fluorescence analysis by Flow Jo software Tree Star, Inc.
FACS analysis by Cell Quest Becton Dickinson
FACSDiva software BD Biosciences
2. Materials and Methods

2.2. Methods

2.2.1. Purification of tumour-initiating cells from patient material

After patients’ consent was obtained, surgical specimens from three selected patients - that were later histologically confirmed to constitute glioblastoma - were dissociated mechanically and taken up in ice-cold PBS followed by five-minute centrifugation at 1300 × g/room temperature. After discharging the liquid, tumour pellets were resuspended in 5 ml TrypsinLE Express and incubated for five minutes. Tumours were then filtered through cell strainers exhibiting a pore size of 70 µm and taken up in DMEM/F-12 medium supplemented with 0.01 % EGF, 0.04 % bFGF, 2 % B27 without vitamin A, 1 % penicillin/streptomycin and 2 % fungizone. In course of about three weeks to three months patient-derived glioblastoma spheres were grown, when medium was exchanged once a week. By swapping the medium for DMEM concentrated with 10 % FCS, 1 mmol/l glutamine, 1 % penicillin/streptomycin, 25 mmol/l HEPES buffer as well as using tissue-culture flasks, adherent cells were obtained within 24 h and subsequently passaged twice a week by using trypsin/EDTA solution for cell detachment. With regard to an altered genomic phenotype when cultured for more than about 12 weeks (De Witt Hamer et al. 2008) this span of cell culturing was not exceeded after first spheroids had appeared.

The study was approved by the Ethics Committee, Medical Faculty, Ulm University.

2.2.2. Changes in cell number

Adherent glioblastoma cells were seeded in 24-well tissue culture plates at a density of 0.5 × 10^5 cells/cm^2, which was tantamount to 1.88 × 10^5 cells/ml provided that each well (1.88 cm^2) was filled with 500 µl medium. For cell counting measurement, medium was removed and 300 µl of Trypsin/EDTA solution were added to each well. After 5 min of incubation time 100 µl of the resuspended cell solution were diluted in 10 ml Casyton liquid and subsequently measured with Cell counter Casy®1.

In case of TiCs also seeded at 1.88 × 10^5/ml, 300 µl TrypsinLE were used to detach cells from each other after medium had been removed by additional 5-min centrifugation at 1300 × g/room temperature.
2. Materials and Methods

2.2.3. Determination of cell death

Quantitative measurement of cell death was performed according to a protocol developed by Nicoletti and colleagues, 1991. Cells were seeded in 24-well plates and allowed to settle overnight before temozolomide or radiation treatment was started. At indicated length of time supernatants were collected and 200 µl trypsin were added to each well. After adherent cells had detached, 400 µl PBS stopped enzyme reaction and following centrifugation at 1300 rpm (4 °C) facilitated resuspension of pelleted cells in 100 µl of hypotonic fluorochrome solution containing 50 µg/ml propidium iodide, 0.1 % sodium citrate and 0.4 % triton-X 100. For want of adherent phenotype TiCs could be exposed to this Nicoletti buffer after a single centrifugation step, only. Incubation time spanned 10 s to 3 h and content of hypodiploid DNA was determined by flow cytometry using fluorescence-activated cell-sorting (FACS). If noted, percentage of specific DNA-fragmentation was calculated as follows: 100 × \[\text{experimental DNA-fragmentation (\%)} - \text{spontaneous DNA-fragmentation in medium-DMSO (\%)}\] / [100 % – spontaneous DNA-fragmentation in medium-DMSO (\%)].

2.2.4. Western blot analysis

Protein extraction and quantification

Each step of protein isolation was performed on ice. 24 h prior to protein isolation cells were seeded in 6 well plates at a density of 1.88 × 10^5 cells/ml. When medium was discharged the next day, adherent tumour cells from several plates were scraped off, collected and subsequently centrifuged in PBS at 1.800 rpm for 5 min. Obtained cell pellets were resuspended in PBS and centrifugation and washing steps were repeated twice. For suspension cells 3 centrifugation steps were performed at 1.300 rpm. Cells were lysed for 30 min on ice in lysis buffer containing 30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % Triton-X, 10 % glycerol, 2 mM DTT and 200 µM PMSF. Ensuing centrifugation at 14.000 rpm for 20 min (4 °C) yielded clear supernatants. Quantification of protein content was determined using BCA™ Protein Assay Reagent Kit and ELISA plate reader according to the manufacturers’ instruction manual.

Gel electrophoresis and blotting

Based on their molecular weight, proteins were separated by means of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS). Therefore, 4 µl of 6 ×
2. Materials and Methods

SDS loading buffer containing 60 mM Tris/HCL pH 6.8, 1 % SDS, 5 % glycerol, 0.01 mg/ml bromphenolblue and 0.34 M β-mercaptoethanol were added to 50 µg protein per sample and filled up to a total volume of 18 µl with H2O. Samples were denatured for 5 min at 95 °C, loaded into 12 % polyacrylamide gels and separated at 150 Volts. Electrophoresis was done in running buffer consisting of 125 mM TrisBase, 1.25 M glycine and 0.1 % SDS. Immediately after electrophoresis, proteins were transferred onto a hybond ECL nitrocellulose membrane by a semidry blotting system at a constant current of 1 mA/cm² for 80 min after thorough moistening with blotting buffer (48 mM TrisBase, 39 mM glyc, 0.037 % SDS and 20 % methanol). Membranes were blocked for 1 h in PBS supplemented with 5 % milk powder and 0.1 % Tween to reduce unspecific binding of antibodies to the hybond ECL nitrocellulose membrane.

Detection of proteins/phospho-proteins
Overnight, membranes were incubated at 4 °C with primary antibody diluted in PBS containing 0.1 % Tween, 2 % BSA and 0.02 % sodium acid. After washing three times with PBS-Tween, incubation for 1 h at room temperature with horseradish peroxidase-coupled secondary antibody diluted in milk followed. Using an ECL kit and Hyperfilm ECL as described in the operating manual, proteins were visualised via enhanced chemoluminescence. For all Western blot analysis β-actin was selected as loading control. Representative data out of at least two experiments are shown.

2.2.5. Fluorescence microscopy
For immunofluorescence staining differentiated glioblastoma cells were seeded in chamber slides at a density of 1.88 × 10⁵ cells/ml and allowed to settle overnight. Double density was chosen in case of TiCs, as over the course of fixation, staining and various steps of washing a vast majority of seeded suspension cells were expected to be rinsed off. At indicated time points cells were washed two times with PBS and fixed with 3.7 % paraformaldehyde for 15 min. After two washing steps with PBS cells were incubated for 15 min in PBS containing 500 nM of MitoTracker-Red. Following two further washing steps with PBS cells were permeabilised with 0.5 % Triton X-100 and reaction was stopped by rinsing the chambers twice with PBS. To block unspecific binding of antibody, cells were incubated with 10 % FCS (in PBS) for 60 min. 200 µl of primary antibody (dilution 1:100) were added to each chamber and incubated at 4 °C overnight followed by four washing steps with 10 % FCS (in PBS). Incubation with secondary antibody (dilution 1:200) for 1 h
2. Materials and Methods

was followed by a fourfold repeated washing step with 10 % FCS. Chambers were removed from the slides and mounting medium was added. Pictures were taken with an AX70 'Provis' microscope.

2.2.6. Reverse Transcription PCR (RT-PCR)

Unless noted otherwise, each step was performed on ice.

Isolation of mRNA from primary glioblastoma cells and determination of RNA concentration

To obtain total cellular mRNA, cells were collected from flasks and pelleted by centrifugation for 5 min at 1800 rpm, 4 °C. Isolation of mRNA was performed by using the RNeasy Mini Kit (Qiagen) according to the manufacturers’ recommendations and RNA concentration was quantified by photometry.

cDNA synthesis

Reverse transcription was carried out applying the ImProm-II™ Reverse Transcription System. Therefore, 1 µg of RNA was mixed with 1 µl Oligo-dT₁₅-Primer and nuclease free water to make up a total volume of 5 µl. After 5 min of incubation at 70 °C and further 5 min at 4 °C following substances were added:

4 µl  
4 × RA buffer

2.4 µl  
MgCl₂ (25 mM)

1 µl  
dNTPs (10 mM)

0.5 µl  
RNAsin-Inhibitor

1 µl  
ImProm-IIRT Reverse Transcriptase

6.1 µl  
nuclease-free water

to a total volume of 15 µl

After centrifugation following temperature steps were run in thermocycler:

25 °C  5 min

42 °C  60 min

70 °C  15 min
2. Materials and Methods

Generated cDNA was stored at -20 °C.

**PCR**

Pursuant to usage information of PCR-Master Mix (Promega), a total volume of 50 µl was made up mixing following components:

- 4 µl cDNA
- 1 µl Forward Primer (10 µM)
- 1 µl Reverse Primer (10 µM)
- 25 µl PCR Master Mix, 2 x
- 29 µl nuclease-free water

After 12 min of denaturation at 95 °C RT-PCR analysis was performed with a Mastercycler gradient (Eppendorf), applying the following thermal profile:

- 28 cycles
  - 94 °C  45 s
  - 55 °C  60 s
  - 72 °C  60 s

ensued by

- 72 °C  10 min
- 4 °C  finish

GAPDH served as control for transcription of equal amounts of cDNA.

**Analysis of cDNA by agarose gel electrophoresis**

For separation of DNA fragments 1 % TAE-agarose gels containing 0.5 µg/ml ethidium bromide and 1 x TAE-running buffer (4.84 g Tris base, 1.14 ml acetic acid, 2 ml 0.5 M EDTA (pH 8) ad 1 l) were used. Samples were supplied with 1/6 volume loading dye and separated by 10 V/cm². As ethidium bromide intercalates into DNA and fluoresces when
2. Materials and Methods

excited by UV-light, gel electrophoresis could be documented using Gel Imaging Intas System.

2.2.7. Examination of glioblastoma cell proliferation by fluorescent cell-staining dye carbofluorescein succinimidyl ester (CFSE)

Carboxyfluorescein succinimidyl ester labeling was performed using the CellTrace™ CFSE cell proliferation kit (Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer’s instructions. Thus, after $2 \times 10^6$ cells had been washed by centrifugation in PBS at 1300 rpm for 5 min room temperature and supernatant had been discarded, cells were resuspended in 1800 µl PBS. 200 µl CFSE (50 µM) were added in order to make up a final concentration of 5 µM. 10 min of incubation at 37 °C followed. To prevent untimely bleaching of the staining dye, all steps were performed considering best possible daylight shielding. Ensuing additional 10 min of incubation time at room temperature after 10 ml of medium had been added, three steps of washing in 8 ml medium and 1300 rpm centrifugation were performed. Finally, cells were seeded in 6-well plates at a density of $1 \times 10^7$/ml, whereas each well was filled with 2 ml. At indicated time points trypsinised adherent glioblastoma cells as well as glioblastoma TiCs were resuspended in 300 µl PBS and intracellular amount of CFSE was determined with BD LSR II flow cytometer using BD FACSDiva software.

2.2.8. Intracranial xenografting of human glioblastoma cells in an orthotopic mouse model

Human glioblastoma cells (G35 TiC, G35 d) were implanted stereotactically into the right temporal lobe of NOD.Cg-PrkdcsidIi2rtm1WjUj/SzJ mice at indicated cell numbers as previously described by Nonnenmacher et al. 2015. Mice were sacrificed upon first manifestation of symptoms like retardation of movement, hemiparesis or scrubby fur. Tumours were extracted, snap frozen and dissolved mechanically followed by trypsination with TrypsinLE for 10 min and several washing steps with PBS. Animal experiments were approved by the representative authorities in Tübingen, Germany. All animal experiments were conducted at animal houses at Ulm University.
2.2.9. Immunohistological staining of murine tissue samples

Tumours were fixed in 4 % paraformaldehyde, embedded in paraffin, cut in 100 µm sections and analysed by immunohistochemistry using 1:1 hematoxylin and 0.5 % eosin. Additionally, staining for human anti-nestin was performed according to the manufacturer’s protocol. Images were digitally recorded at indicated magnification using an AX70 microscope.

2.2.10. Statistical analysis

Statistical significance was carried out by a two-sided Student’s t-Test. Statistical significance level was determined as significant at p < 0.05.
3. Results

3.1. Extraction of conjectural tumour-initiating cells from clinical tumour samples

After by half of tumour material from three selected patients suffering from histologically proven glioblastoma had been stored as reference at -80 °C, the remaining bulk residues were passed through further steps of processing that in short spanned mechanical and enzymatic disassociation, continuative purification and following expansion when cultured in DMEM/F-12 medium lacking adherent substrate FCS, but supplemented by B27, hEGF and hFGF (Figure 5; for details see Materials and Methods). In course of about three weeks to three months free-floating conglobations of spherical-appearing cells were come upon that, referring to De Witt Hamer and colleagues, were supposed to retain the genomic profiles resembling those of their parental tumours when still cultured as spheroids. Swapping medium for DMEM concentrated with FCS and L-glutamine as well as using tissue-culture flasks induced suspension cells to alight on the bottom of the flask accompanied by contracting an adherent phenotype. In the present study, these putative differentiated glioblastoma cells ought to get more characterised with regard to some key features of tumour biology in order to permit a paired comparison to those of their undifferentiated counterparts. All along more than ten population doublings were avoided in the view of an altered genomic phenotype when cultured for over 12 weeks after first spheroids had emerged (De Witt Hamer et al. 2008).
3. Results

Figure 5: Overview of the isolation of putative tumour-initiating cells from clinical tumour samples
For details see text.
Modified from Schneider et al. 2016 with permission from International Journal of Cancer.
The data 35, 38 and 40 were attributed arbitrarily to the primary material isolated from the three tumour samples, mentioned above, whereat the prefixed letter ‘G’ signifies an acronym for glioblastoma. Hence, regarding conjectural tumour-initiating cells (TiCs) together with their differentiated progeny, abbreviated as ‘d’, three pairs of glioblastoma cell populations were taken as a basis of ensuing experiments.

All three tumour samples have proven to constitute malignancies alleged TiCs could be extracted from. In any case, isolated suspension cells formed into characteristic 3-D clusters that were redolent of the spherical formation of neural progenitor and stem cells first isolated from adult mice brain tissue by Reynolds and Weiss in 1992 (Reynolds and Weiss 1992) (Figure 6). By comparison with the two remainder primary suspension cell lines, G38 TiCs exhibited a propensity for engendering spheroids of major diameter. Since cells could be detached from each other by only few resuspension steps, less intraspheroid cohesion was assumed for G35 TiCs.

In the case of differentiated progeny, some potential morphologic distinctions had us to gather from light microscopy images. While G35 d cells displayed rather an acicular pattern showing a rectilinear axis passing through the two only and properly opposite cell protrusions (Figure 6A), G40 ds comprised a conspicuous number of astral-appearing cells that occasionally exhibited up to five, although less lathy protrusions (Figure 6C). According to this, G38 d morphology was about halfway between these two cell populations, as they included spicular as well as slightly branched cells in about equal shares (Figure 6B).

However, it has to be mentioned that this description based on light microscopic aspects is the matter of an obvious simplification, because each morphologic token can also be found among the other differentiated cell populations, even though to another ratio.
3. Results

Continuation on next page
3. Results

Continuation of Figure 6

Figure 6: Light microscopic images of conjectural tumour-initiating cells and their differentiated progeny isolated from three patients suffering from glioblastoma
A) G35 cell populations B) G38 cell populations C) G40 cell populations. Pictures were taken during the early stages of established cell culture.
Magnification: left 10 ×; right 40 ×.
d, differentiated glioblastoma cells; G, glioblastoma; TiC, tumour-initiating cells.
Notwithstanding observed spherical formation of cells could be considered a possible indication of TiCs, expression of nestin was examined with regard to further verification, as the said intermediate filament protein can be detected in the majority of mitotically active CNS and PNS progenitors that give rise to neurons and glial cells. While nestin is expressed during the early stages of development in the CNS and other tissues, terminal differentiation processes involve down-regulation of nestin as well as replacing induction of tissue-specific intermediate filament proteins that include GFAP in case of astrocytes. Therefore, nestin has been widely used as a marker for stem cells that characteristically feature multipotency, self-renewal and regeneration, though little is known about its function and regulation (Michalczyk and Ziman 2005).

Immunofluorescence staining of nestin, depicted in Figure 7A, was stronger and more distinct in suspension cells, whereas adherent progeny did not show consistent strong visual signals. Detection of differentiated astrocyte marker GFAP revealed inverse findings, as positive dotted staining was particularly present in adherent, but scarcely in suspension glioblastoma cells (Figure 7B). Therefore, we could suppose to have succeeded in isolating three glioblastoma suspension cell populations that were markedly enriched in common stem cell marker-positive cells. As stem cell marker-expressing tumour cells are generally assumed to be prerequisite for tumour initiation in immunocompromised mice (Cruceru et al. 2013, Baccelli and Trumpp 2012), we referred to these cell populations as tumour-initiating cells (TiCs) or cancer stem cells (CSCs), hence.
3. Results

**Figure 7: Immunofluorescence staining for stem cell/differentiation markers**
Differentiated glioblastoma cells were seeded at a density of $1.88 \times 10^5$ cells/ml. Double density was chosen in case of TiCs. Tumour cells were stained for either A) the stem cell marker nestin or B) the differentiation marker GFAP. One representative experiment out of two is shown. Magnification 40 x.

d, differentiated glioblastoma cells; G, glioblastoma; GFAP, glial fibrillary acidic protein; TiC, tumour-initiating cells.
3. Results

3.2. Glioblastoma TiCs and their differentiated descendants predominantly featured different proliferation rates

Comparable to many adult stem cell types, quiescent and slow-cycling states have also been reported for several CSCs (Wilson et al. 2008, Essers et al. 2009), which thereby could be equipped with an intrinsic resistance to conventional therapies designed to kill rather rapid cycling cells (Clarke et al. 2006).

Against this background, we first focused on putative proliferation distinctions between glioblastoma TiCs and their differentiated counterparts. Therefore, initially about equal numbers of seeded cells were matched over a period of 72 h. Indeed, all three pairs of glioblastoma cell populations revealed TiCs lagging behind the quantity of their adherent descendants: G35 TiCs and G38 TiCs roughly kept to the cell number seeded at 0 h, G40 TiCs anyway were able to nearly reduplicate within three days (Figure 8A). In contrast, a distinctly higher cell count of about $6 \times 10^5$ that was even surpassed by G38 ds could be detected for differentiated progeny G35. Even though exceeding the quantity of G40 TiCs at each time of measurement, G40 ds only reached a number of about $5.5 \times 10^5$. As a mere cell number study taken by itself does not support conclusions on proliferation, DNA-fragmentation rates were ascertained additionally according to a protocol of Nicoletti and colleagues (Nicoletti et al. 1991) (Figure 8B). However, with virtually equal DNA-fragmentation rates of all six primary cell lines that roughly persisted at a 10 % - level of day one over the 72 h studied, assertions on cell count obtained from Figure 8A could also be adopted for proliferation, hence.
Figure 8: Glioblastoma TiCs and ds markedly differed in proliferation celerities
Cells were seeded with $1.88 \times 10^5$ cells/ml. A) Cell number was measured at indicated time points using Cell counter Casy®1. B) Percentage of DNA-fragmentation of propidium iodide-stained nuclei was determined by FACS analysis. Mean and standard deviation of three independent experiments performed in triplicate are shown.
d, differentiated glioblastoma cells; FACS, fluorescence-activated cell sorting; G, glioblastoma; TiC, tumour-initiating cells.
3. Results

In order to confirm and further qualify the findings deduced from Figure 8 by means of another methodical approach, CFSE-staining was performed additionally. Representative graphs out of nine measurements taken for each cell population were depicted in Figure 9A.

Among differentiated primary cell populations, G35 ds and G38 ds featured a high proliferation rate, as the graphs of CFSE-stained cells (blue) visualised a fluorescence intensity that in a large part matched the one of unstained cells (red) indicating quite a number of CFSE fluorescence halvings within daughter cells following each cell division. In comparison, G40 ds mainly exhibited a higher amount of intracellular CFSE and therefore less cell divisions could be implied when also measured 120 h after staining. Accordingly, G40 ds again proved to represent the least proliferative adherent cell population of the three examined.

In contrast, discernible peaks of CFSE-stained TiCs denoted a higher CFSE fluorescence at day five after staining compared to respective differentiated cells. However, G38 TiCs and G40 TiCs exhibited a substantial percentage of cells, the fluorescence of which did not match the one of the peak. Apparently, proliferation of G38 TiCs and G40 TiCs proceeded less synchronised with admittedly a considerable amount of high fluorescent cells that barely proliferated, but also a high-grade proliferating subpopulation displaying a CFSE fluorescence that was projected under the red curve of isotype control. This subpopulation was mostly marked within G38 TiCs, but slightly present for G35 TiCs, which in turn featured a more clearly definable peak.

By averaging over the differences between median fluorescent intensities of CFSE-stained cells and isotype control that accrued from nine data, respectively, mean fluorescent intensities (MFIs) of TiCs and differentiated cells could be contrasted in a graph for each paired cell population (Figure 9B). Albeit fluorescent detection was performed after 120 h after staining and proliferation (Figure 8) was examined over 72 h only, the findings deduced from Figures 9B and 8 almost completely coincided. Significantly higher MFIs of all three primary tumour-initiating cell populations compared to adherent cells indicated less cell divisions within five days of investigation time, whereby G40 TiCs proved to represent the most proliferative suspension cell population. Among adherent progeny, a MFI of roughly 212 for G38 ds was linked to the highest proliferation rate, while proliferation of G40 ds lagged behind displaying a MFI of about 400.
3. Results

A)

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G35 TiC

G35 d

G38 TiC

G38 d

G40 TiC

G40 d

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Continuation on next page
3. Results

Continuation of Figure 9

**Figure 9**: Examination of glioblastoma cell proliferation by fluorescent cell staining dye CFSE

Cells were seeded at a density of $1 \times 10^4$ cells/ml. CFSE fluorescence was determined 120 h after staining by flow cytometric analysis. A) Representative graphs out of three independent experiments performed in triplicate are shown. B) Mean fluorescent intensities of TiCs and ds are depicted for each paired cell population.

CFSE, carbofluorescein succinimidyl ester; d, differentiated glioblastoma cells; G, glioblastoma; TiC, tumour-initiating cells.

As a result, slow-cycling states could be ascribed to a large percentage of glioblastoma TiCs isolated from the three patients' tumours. However, a highly-proliferating subpopulation could be detected within G38 TiCs and G40 TiCs conveying that quite a number of cell divisions could not invariably be considered an exclusion criterion for a glioblastoma cell to rank among the tumour-initiating cell population.
3.3. Glioblastoma TiCs did not exhibit lower DNA-fragmentation rates in response to irradiation or administration of temozolomide compared to glioblastoma ds

Particularly with regard to the growing interest in considered publications that proposed an intrinsic resistance to conventional therapies to constitute an important contribution of TiCs to tumour bulk reformation following clinical recovery after initial therapeutical shrinking of the tumour mass, we next tried to compare the impact of glioblastoma standard treatment on TiCs and differentiated glioblastoma cells. Therefore, according to a protocol of Nicoletti and colleagues (Nicoletti et al. 1991), DNA-fragmentation rates were analysed 120 h after cells had been irradiated at a dose of 8 Gy (Figure 10A) or rather treated with 10 $\mu$M temozolomide (TMZ) (Figure 10B). Indicated concentration of TMZ was specifically selected to reflect a low value of the concentrations that had been achieved in the cerebrospinal fluid of patients with glioblastoma undergoing treatment with TMZ (Ostermann et al. 2004, Blough et al. 2010).

With the exception of G38 cells that even showed a significantly ($p < 0.05$) higher DNA-fragmentation percentage for suspension cells, no significant difference between paired counterparts G35 and G40 could be detected in response to irradiation. In a similar way, distinctions of DNA-fragmentation rates after administration of TMZ did not meet the pre-assigned definition of statistical significance ($p < 0.05$) except for G40 cell populations, where TiCs turned out to be more susceptible to alkylating agent-caused DNA-damage. Even against the backdrop of an unknown MGMT promoter methylation status that however was not reported to switch in the course of differentiation processes, we could assume that the GCSCs used did not feature any more pronounced intrinsic mechanisms to encounter radiochemotherapy by lower DNA-fragmentation rates as against adherent cells when measured five days after treatment.
3. Results

Figure 10: Glioblastoma TiCs did not show lower DNA-fragmentation rates after standard treatment modalities when compared to corresponding differentiated tumour cells. Cells were seeded at a density of $1.88 \times 10^5$ cells/ml and allowed to settle overnight. Subsequently, treatment was performed with A) 8 gy of $\gamma$-radiation from a Caesium 137 source and B) 10 $\mu$M of temozolomide. Percentage of DNA-fragmentation of propidium iodide-stained nuclei was determined by FACS analysis 120 h after treatment. Mean and standard deviation of three independent experiments performed in triplicate are shown.
d, differentiated glioblastoma cells; FACS, fluorescence-activated cell sorting; G, glioblastoma; TiC, tumour-initiating cells.
3. Results

3.4. Glioblastoma TiCs and ds distinctly differed in several expression profiles of proteins involved in mediating apoptosis

Having found no clear differences in cell death sensitivity between TiCs and Gds (Figure 10), we next investigated the expression of pro- and antiapoptotic BCL-2 family proteins, as these proteins are key mediators of treatment induced apoptosis and their pharmacological targeting is considered a promising therapeutic approach (Czabotar et al. 2014). Opposing results were obtained for pro- and antiapoptotic BCL-2 family members. Thus, intracellular quantity of proapoptotic proteins BAX, BAK, BAD and BID examined for GCSCs markedly exceeded that detected within flask-attaching glioblastoma cells (Figure 11A). Only in case of proapoptotic player PUMA, observed disparities did not come off that distinctly, as its bright bands additionally appeared quite dimly. Interestingly, detection of the antiapoptotic BCL-2 repertoire BCL-2, BCL-xL and MCL-1 yielded closely inverse findings except for G38 ds, by which protein expression of BCL-xL did not surpass that of G38 TiCs (Figure 11B). Though a mere evaluation of protein expression profiles could not be considered sufficient to be indicative of the intricacy of intracellular pathways, TiCs still seemed to display an at least BCL-2 family manifested priming towards apoptotic signalings, from which an irreversibility could not be reasoned yet, because of an unvetted localisation and activation state of depicted proteins. Viewed in isolation, these data would suggest that Gds are more resistant to apoptosis-inducing treatment than glioblastoma TiCs. However, as this was not the case (Figure 10), we looked for other mechanisms that might influence the cells' resistance towards treatment.

As the PI3K/AKT pathway was reported to highly rank among mutated signalings in human cancer (Engelman et al. 2006) and to interfere in apoptosis regulation via phosphorylation of distinct BCL-2 family proteins among other mechanisms (Peso et al. 1997, Yamaguchi and Wang 2001), Western blot analyses of some key proteins within this pathway were performed and outlined in Figure 11C. While quantities of protein expression of AKT in TiCs visibly surpassed that of ds, protein expression profiles of both its activated forms - pAKT (Ser473) and pAKT (Thr308) - did not evince any obvious differences among the six primary cell populations. In contrast, downstream-emerging ribosomal proteins S6 and pS6 involved in promoting protein synthesis and cell proliferation displayed quite inverse profiles to each other with a high expression of S6 in case of TiCs.

Figure 11D outlines Western blotting of caspase-3, the protein profiles of which were strongly evocative of that seen for proapoptotic BCL-2 family players. Since caspase-3 cleavage products could not be detected and caspase-3 as an effector caspase was said to
be immediately involved in apoptosis induction, but DNA-fragmentation rates of TiCs and ds did not differ in a way one could suppose according to the differences in caspase-3 protein expression (see Figures 8B and 11D), an inactive form of detected caspase-3 was assumed.

After detection of α-tubulin had revealed a marked superior protein expression for glioblastoma TiCs (Figure 11D), β-actin was selected to serve as loading control.

Regarding Figure 11 against the backdrop of the findings deduced from the previous two graphs, one could suppose that, although TiCs and their differentiated progeny surprisingly did not remarkably differ in DNA-fragmentation rates in response to irradiation or administration of temozolomide, they might draw on different cellular mechanisms to counter these therapeutical modalities.
3. Results

A) G35 TiC | G35 d | G38 TiC | G38 d | G40 TiC | G40 d

- BAX
- BAK
- BAD
- BID
- PUMA
- β-actin

B) G35 TiC | G35 d | G38 TiC | G38 d | G40 TiC | G40 d

- BCL-2
- BCL-xL
- MCL-1
- β-actin

C) G35 TiC | G35 d | G38 TiC | G38 d | G40 TiC | G40 d

- pAKT (pS)
- AKT
- β-actin
- pS6
- S6
- β-actin

Continuation on next page
3. Results

Continuation of Figure 11

Figure 11: Expression profiles of apoptosis-regulating proteins
Protein expression profiles were performed by Western blot analysis and compiled to A) proapoptotic BCL-2 family members, B) antiapoptotic BCL-2 family proteins, C) proteins involved in PI3K/AKT signalling as well as D) others. β-actin served as loading control. One representative experiment out of two is shown.

BAD, BCL-2 antagonist of cell death; BAK, BCL-2-antagonist killer; BAX, BCL-2-associated X protein; BCL-2, B-cell-lymphoma-2; BCL-xL, B-cell-lymphoma-extra large, BID, BCL-2-interacting domain death agonist; d, differentiated glioblastoma cells; ERK, extracellular regulated kinase; G, glioblastoma; MCL-1, myeloid cell leukemia sequence-1; pERK, phospho-ERK; pS, phospho-serine; pS6, phospho-S6; pT, phospho-threonin; PUMA, p53 upregulated modulator of apoptosis; S6, S6 ribosomal protein; TiC, tumour-initiating cells. Modified from Schneider et al. 2016 with permission from International Journal of Cancer.
3. Results

3.5. Immunofluorescence staining of MCL-1, BID and BAD confirmed Western blot analyses on a single-cell level and predominantly revealed a mitochondrial localisation of these proteins

Hereafter, we further focused on our detections respecting BCL-2 family expression profiles. In order to approve the findings of Figure 11A by use of another methodical approach as well as to concurrently consider the question of intracellular protein localisation, immunofluorescence staining of selected family members was performed and analysed with regard to potential distinctions within the three pairs of glioblastoma cell populations, respectively.

As MCL-1, BID and BAD were reported to predominantly function when localised around or within the outer mitochondrial membrane (OMM) (for details see introduction), staining of these proteins was complemented by imaging of mitochondria by MitoTracker-Red and subsequently presented as an overlay in Figure 12.

In keeping with Figure 11B, particularly differentiated glioblastoma cells revealed specific green visual signals for MCL-1 that were additionally projected onto red-phosphorescing mitochondria in the overlay, now (Figure 12A) indicating a preponderant mitochondrial localisation of MCL-1 within adherent cell populations G35 d, G38 d and G40 d. As inverse findings for detection of the proapoptotic proteins BID (Figure 12B) and BAD (Figure 12C) could be detected for G35 paired cell populations displaying a mitochondrial localised positive green-dotted staining especially for TiCs, Western blot analyses (Figure 11) were distinctly approved on a single-cell level in these attempted cases.

Over the course of fixation, staining and various steps of washing, a vast majority of originally seeded suspension cells were rinsed off resulting in only few stained TiCs that were on hand in order to contrast with comparatively plenty of Gds. Besides, due to a shifting of free-floating TiCs caused by compression of the object slide by the lens, overlays in case of BID and BAD staining were relinquished, as respective pics did not project onto corresponding mitochondria-visualising images.
3. Results

A)
3. Results

Continuation of Figure 12

A)

B)

Continuation on next page
Figure 12: MCL-1, BID and BAD mainly revealed a mitochondrial localisation
Cells were seeded on glass slides and allowed to adhere overnight. MitoTracker-Red was used to visualise mitochondria (red) followed by staining of A) MCL-1, B) BID as well as C) BAD (green). In some cases, mitochondria- and protein-visualising overlays are shown. Stainings were analysed by fluorescence microscopy. Representative results of two independent experiments are depicted. Magnification: 40 ×.
BAD, BCL-2 antagonist of cell death; BID, BCL-2-interacting domain death agonist; d, differentiated glioblastoma cells; G, glioblastoma; MCL-1, myeloid cell leukemia sequence-1; TiC, tumour-initiating cells.
3. Results

3.6. **Observed protein expression profiles of BID could be confirmed on mRNA level in case of matched primary G38 cell lines**

In order to ascertain at which level of the translational machinery the observed differences occur, i.e. are we dealing with a phenomenon of differential gene expression or differences in protein stability/turn over, reverse transcription PCR of two selected BCL-2 family members was performed for two matched pairs of glioblastoma cell populations. Therefore, we chose BID, the protein expression profile of which revealed marked distinctions between TiCs and ds (Figure 11A, 12B), as well as the pro-apoptotic BCL-2 family member Noxa, which for technical reasons could not be detected persuasively in the Western blot analysis.

As shown in Figure 13, mRNA expression of both BID and Noxa detected for G38 GCSCs exceeded that of corresponding differentiated tumour cells, whereby cDNA levels served as surrogate for mRNA expression. These findings might indicate that observed differences in intracellular protein quantity of BID (Figure 11A and 12B) can be explained on transcriptional level. However, similar observation could not be seen in case of matched G35 primary cell populations.
3. Results

Figure 13: mRNA expression profiles of proapoptotic BCL-2 proteins BID and Noxa for matched G35 and G38 cell population
A) Cells were collected from flasks and RT-PCR mRNA expression was performed. GAPDH served as loading control. One representative experiment out of two is shown.
B) Densitometric analysis of a single, representative experiment normalised to GAPDH expression. Different cDNA-levels served as surrogate for relative mRNA expression.
BCL-2, B-cell-lymphoma-2; d, differentiated glioblastoma cells; BID, BCL-2-interacting domain death agonist; G, glioblastoma; GAPDH, glycerinaldehyde 3-phosphate dehydrogenase; Noxa, Latin for damage; TiC, tumour-initiating cells.
3.7.  Glioblastoma TiCs and ds revealed similar tumour-initiating capabilities

Notwithstanding the growing number of publications in the field of CSCs, till date the only agreed upon defining characteristic of TiCs has remained their ability to propagate and recapitulate the complexity and heterogeneity of the patient’s tumour when engrafted into immunodeficient mice at low cell numbers (Beck and Blanpain 2013). Along these lines in a study of Singh and colleagues, as few as 100 CD133⁺ GCSCs were sufficient for the formation of human brain tumours in NOD-SCID mice, whereas 50,000 to 100,000 of CD133⁻ cells engrafted, but did not cause a tumour (Singh et al. 2004). In our setting using an orthotopic mouse model, we selected G35 TiCs and ds to analyse in view of putative distinctions in tumour-initiating capabilities, when 500 or 100,000 suspension respectively matched differentiated glioblastoma cells were injected into the rodents’ temporal lobes. After cells had been transplanted at day zero, survival data were ascertained and compiled to Figure 14. Each group was comprised of three animals, whereas for G35 d high, i.e. injection of 100,000 differentiated G35 cells, survival of only two mice could be analysed, as one rodent died two days after transplantation probably due to technical difficulties. Apart from that, all animals of the groups with low numbers of transplanted cells (G35 TiC low, G35 d low) featured prolonged survival data when compared to G35 TiC high and G35 d high. Seen over the observation period of 80 days, one rodent in group G35 TiC low survived. However, it appeared that - especially in case of the groups ‘low’ - injection of ds resulted in less days of survival.
3. Results

Figure 14: Survival data after injection of glioblastoma cells in an orthotopic mouse model

Cells were transplanted at day zero. Except the demise of one rodent at day two in group G35 d high presumably due to technical difficulties, all groups were made up of three animals indicated with special signs, respectively.

High, injection of 100,000 cells; low, injection of 500 cells; d, differentiated glioblastoma cells; G, glioblastoma; TiC, tumour-initiating cells. Modified from Schneider et al. 2016 with permission from International Journal of Cancer.

In addition to survival data indicating death-causing tumour formation for both G35 TiCs and G35 ds, mice brains subsequently were dissected and examined via H.E. staining. In keeping with abovementioned supposition deduced from Figure 14 and with the exception of the rodent that survived over the 80 days of observation time, all animals of the groups examined (TiCs versus ds, 500 versus 100,000 initially implanted cells) showed clearly visible, highly cellular tumours upon histological analysis (examples shown in Figure 15). Occasionally necrotic foci were detected, but no pseudo-palisading of tumour cell nuclei was found. Regarding the representative extracts on the right in Figure 15A and 15B, respectively, tumours derived from both initially implanted CSCs and differentiated glioblastoma cells seemed to exhibit a comparable micrometastatic phenotype, as an about equal number of pleomorphic, hyperchromatic nucleated cells broke the border of the tumour masses migrating into the adjoining tissue.
Figure 15: Both injection of glioblastoma TiCs and ds yielded formation of tumorous masses in an orthotopic mouse model

G35 TiCs A) respectively ds B) were transplanted at a number of 500 into the temporal lobes of immunosuppressed mice. Upon sacrificing the animals, brains were dissected and analysed via H.E. staining (experimental results from injection of 100,000 cells are not shown). Images reveal tightly packed tumour cells growing as a disordered malignant mass, respectively. Magnification: left 2 ×; middle and right 10 ×.

d, differentiated glioblastoma cells; G, glioblastoma; H.E., hematoxylin and eosin; TiC, tumour-initiating cells.

Thus, adherent G35 cell population that had proven to be markedly enriched in differentiation marker GFAP-positive cells (Figure 7) and seemed to also be capable of tumour initiation even when transplanted at low cell numbers consequently raises to question whether for the malignancy of glioblastoma tumour formation initiated by a comparative less number of tumour cells might represent a special distinguishing feature of TiCs and ds, therefore whether the synonym tumour-initiating cell can live up to the term of cancer stem cells or even beyond whether GCSCs constitute the priority cell population for tumour initiation, maintenance and propagation - all of which are characteristics commonly related to CSCs.
3. Results

3.8. Tumours exhibited nestin-positive glioblastoma cells irrespective of their origination from suspension or adherent tumour cells

Finally, tumours obtained from the abovementioned orthotopic animal experiment were additionally stained for the stem cell marker nestin. Just like in case of tumours induced by transplantation of G35 suspension cells (Figure 16A), a comparably low number of nestin-positive tumour cells could also be detected for those initiated by G35 cell population, that had been shown to be markedly enriched in GFAP-positive, nestin-negative tumour cells (Figure 16).

While it is possible that sufficient GCSCs were present in the Gd-enriched cell population to initiate tumour formation, the difference in total GCSCs present should have been reflected in a lack of Gd-derived tumours reaching a critical mass. However, as this was not the case (Figure 14), these results might point to a rather bi- than unidirectional hierarchy concerning CSCs and differentiated tumour cells, which offers the additional possibility of dedifferentiation processes that might reprogram differentiated tumour cells into stem-like cancer cells within a single tumour.
3. Results

**Figure 16:** Both transplantation of G35 GCSCs and ds resulted in tumorous masses that exhibited a comparably low number of occasional nestin-positive tumour cells in an orthotopic mouse model.

G35 GCSCs A) respectively G35 ds B) were transplanted at a number of 500 into the temporal lobes of immunocompromised mice (experimental results from injection of 100,000 cells are not shown). Upon sacrificing the rodents, brains were dissected and stained for the stem cell marker nestin (auburn). Nuclei were counter-stained in blue. Arrows in the highlighted selections indicate examples of nestin-positive cells. Magnification: 20×.

d, differentiated glioblastoma cells; G, glioblastoma; GCSCs, glioblastoma cancer stem cells.
4. Discussion

In order to investigate whether several frequently posited key perceptions concerning the so-called concept of cancer stem cells could also be extended to the malignancy of glioblastoma, this study was based on three matched pairs of putative cancer stem cells and their differentiated descendants isolated from three human glioblastoma tumour specimens. We focused on comparative experiments regarding conjectural differences in proliferation, resistance to radiochemotherapy as well as protein expression of apoptosis-mediating key players. Finally, tumour-initiating capabilities of one matched pair of glioblastoma cell populations were examined in an orthotopic mouse model.

4.1. Determination of purity of glioblastoma TiCs and differentiated glioblastoma cells

When establishing and characterising the cell populations used in this study, putative stem cells were first isolated by culturing cells disassociated from the tumour in a serum-free suspension culture supplemented by EGF and bFGF, i.e. under conditions optimised for neural stem cells. This is an acceptable method used within the research community to obtain cancer stem cells (Reynolds and Weiss 1992, Tirino et al. 2012) and has also been successfully used for glioblastoma (Yuan et al. 2004). Cells were then provoked into differentiation by letting them adhere in a serum-enriched environment, allowing us to create paired populations of tumour-initiating cells (TiCs) and corresponding differentiated cells (ds). Importantly, as mentioned in 3.1., these adherent cells loose their distinct expression profile when cultured for prolonged periods of time, while suspension cells growing in spheres are frequently less deviant from parental tumour samples (De Witt Hamer et al. 2008). Along these lines established glioblastoma cell lines are not only unlikely to represent a gene expression profile similar to their tumour of origin, they are frequently not even found to represent glioblastoma profiles in general (Li et al. 2008, Vogel et al. 2005). Therefore, we have completely avoided the use of established cell lines. In order to further characterise the CSCs and differentiated cells and to verify that indeed the suspension culture is sufficient to select for ‘stemness’, while adherent culturing conditions lead to differentiation, we decided to additionally confirm cell purity by the relative expression of selected stem cell and differentiation markers. Since intermediate filament protein nestin has been found to be expressed in neural progenitor/stem cells during the early developmental stages of the CNS (Lendahl et al. 1990), nestin has been shown to also serve as a cancer
stem cell marker in the field of tumour biology, such as glioblastoma (Jin et al. 2013, Binello et al. 2012). Because of additional reports indicating nestin downregulation in the human embryonic midbrain stem cell line NGC-407 after induction of differentiation (Khan et al. 2011) as well as differentiation-associated downregulation of nestin and its replacing induction by tissue-specific intermediate filament proteins, such as GFAP in case of astrocytes (Michalczyk and Ziman 2005), we finally compared alleged TiCs and thereof differentiated tumour cells by means of immunofluorescence staining for nestin and GFAP. Restrictively, one should annotate that down-regulated nestin might also be re-expressed in the adult organism under neoplastic transformation (Zhang et al. 2008). However, since nestin expression was markedly associated with spherical-growing, but scarcely therefrom emerging differentiated GFAP-positive glioblastoma cells, nestin was ascribed to a stem cell-like phenotype in our setting.

Of note, we have avoided the use of the CD133 marker protein that has been taken as a basis of CSC detection in numerous publications since cell surface expression of the human CD133 antigen has been suggested a novel marker for stem and cancer stem cells (Yin et al. 1997, Grosse-Gehling et al. 2013, Uchida et al. 2000). In terms of glioblastoma, Singh et al. compared the ability of CD133\(^+\) and CD133\(^-\) tumour cells to initiate tumour formation in NOD-SCID mice. Indeed, only CD133\(^+\) cells yielded tumours that could be serially transplanted, while injection of CD133\(^-\) cells engrafted, but did not produce a tumour (Singh et al. 2004). However, newer data have questioned this approach by demonstrating that at least a subgroup of glioblastoma is driven by CD133\(^-\) TiCs (Beier et al. 2007, Ogden et al. 2008). The observation that tumours induced by CD133\(^-\) cells in mice even exhibited CD133\(^+\) cells supported the idea, whereupon CD133 expression might be acquired after xenotransplantation, but was not required for brain tumour initiation (Wang et al. 2008). Similar conclusions were drawn, when glioma-initiating cell lines were induced by overexpressing both SV40 large T antigen and a constitutive active form of H-Ras in mouse neural stem cells, the CD133 expression of which could be eliminated by tamoxifen-dependent Cre activation. This induced glioblastoma-initiating cell population has been shown to form tumour spheres in culture and transplantable glioblastoma in vivo (Nishide et al. 2009). As therefore CD133 did not appear to be essential for stem cell-like properties (Beier et al. 2011), we did not use an approach that involved cell sorting by surface markers, but decided to confirm cell purity by the relative expression of selected, abovementioned stem cell and differentiation markers.
4. Discussion

Thus, we could confidently assume primary cell lines of different stages of differentiation to have taken as a basis of further experiments.

4.2. Examination of cell proliferation revealed unsynchronised-cycling TiCs in case of G38 and G40 tumour specimens

Regarding the capability of self-renewing, differentiation and reconstitution of the original tissue, when injected into mice, CSCs seem to feature several key traits of normal tissue stem cells. Accordant to this assumption, molecular profile analyses of GCSCs have recently revealed a range of molecular markers to be also possessed by adult stem cells (Liu et al. 2009). Against the backdrop of a conceivable origination of CSCs from normal SCs as outlined in the introduction, these closely related properties are nearly bound to occur. Thus, CSCs could be equipped with some more key stem regulatory traits that might decisively contribute to tumour biology. Slowly-dividing or even dormant states rank high among these stem cell characteristics as they have been reported for the hematopoietic system (Wilson et al. 2008), the prostate (Tsujimura et al. 2002) and the brain (Morshaed et al. 1998) among others. We therefore decided to contrast growth characteristics of TiCs and differentiated tumour cells within the three matched pairs of human glioblastoma cell populations used in this thesis.

Indeed, our data also indicate a high percentage of TiCs in glioblastoma featuring a distinct lower proliferation rate than corresponding differentiated cells. All three tumour specimens have proven to exhibit such slowly-cycling TiCs. However, in case of G38 and G40 TiCs, measurement of CFSE fluorescence intensity that had been repeatedly used to identify slowly-cycling, label-retaining cells in several cancers (Moore et al. 2012, Deleyrolle et al. 2011) even revealed a barely fluorescing subpopulation indicating a number of cell divisions that did not rank behind those of G38 and G40 ds. Additionally, both these primary cell lines exhibited less synchronised proliferation properties when compared to matched differentiated tumour cells (Figure 5).

Consistent with our results there is quite a plenty of publications providing evidence for quiescence to also play a role in cancer stem cells besides its existence in normal tissue stem cells as mentioned above. Using an orthotopic model of hepatocellular carcinoma in NOD/SCID/Il-2 rγ null mice, Martin-Padura and colleagues found that metronomic administration of cyclophosphamide eradicated the tumour mass, but a residual population of dormant cells could be found yet accounting for tumour regrowth after discontinuation of therapy (Martin-Padura et al. 2012). Obviously, these dormant CSCs were able to re-enter...
Discussion

into a growing phase of cell division and regenerate tumour growth. Similarly, in ovarian cancer, a subset of CD24$^+$ cells that were positive for expression of stem cell genes, such as nestin, Oct-4, notch1 and notch4, proliferated slowly compared with the CD24$^-$ fraction suggesting a quiescent phenotype (Gao et al. 2010). In a study of Dembinski and Krauss, a subpopulation of slowly-proliferating stem cell-like cells in pancreatic adenocarcinoma cell lines was identified using long term lipophilic tracer dye DiI in a label retention technique. These cells exhibited a tenfold increase in colony formation, a twofold increase in invasive potential as well as more than a tenfold increase in tumourigenic potential when compared to the DiI$^-$ cell population (Dembinski and Krauss 2009). Notwithstanding most publications have closely linked the term of CSCs to quiescence and dormancy as set out above, in present times some tissues and cancers have also been reported to harbour more rapidly-dividing SCs and CSCs, respectively. In a study of Driessens et al., cell fate of selected cells as well as of their descendants could be traced over time at different stages of tumour progression by using a genetic labelling strategy that made tumour cells express yellow fluorescent protein. While most tumour cells in benign skin tumours featured limited proliferative potential, a minority was capable of persisting long term and dividing rapidly as well as giving rise to posterity that mainly constituted the tumour bulk. Quantitative analysis of clonal fate data supported evidence for the existence of rapidly-cycling CSCs (Driessens et al. 2012). Barker and colleagues have detected distinct columnar cells at the crypt base of human small intestine and colon that generated all epithelial lineages implicating intestinal stem cell characteristics. These cells were frequently positive for proliferation marker Ki67 and the M-phase marker phospho-histone H3 indicating that the cells were actually cycling. The average cycling time was dated for about one day (Barker et al. 2007). Examining the proliferative status of CSCs in primary and recurrent medulloblastoma, a subset of nestin and CD133 expressing cells was also positive for Ki67 antigen pursuant to a proliferative phenotype (Tang et al. 2011). These studies show surprising resemblance to our data of a highly proliferating subpopulation concerning G38 and G40 TiCs, even though we did not look for Ki67 expression. By implication, temporal coexistence of GCSCs that do not all share the same state of proliferation within a single tumour, seems more than likely. According to this, it is rather the ability to reversibly enter a quiescent or dormant state and not the exclusive existence in such a state, which might be considered a defining feature not only of SCs, but also of CSCs. Even though in the view of bone marrow injury, hematopoietic stem cells (HSCs) forming a silent reservoir during homeostasis have been shown to efficiently get activated accompanied by wakening from
dormancy and entering a state of faster cell divisions (Wilson et al. 2008). Besides, in response to treatment of mice with interferon-α, transplanted human HSCs efficiently exit G₀ and enter an active cell cycle (Essers et al. 2009). The transcriptional repressor Hairy and Enhancer of Split1 (HES1) has been shown to maintain quiescence to be reversible, as it prevented both premature senescence and inappropriate differentiation in dormant fibroblasts. Increased expression of HES1 has been observed in several tumours, including meningiomas and medulloblastomas, as well as in stem-like cells isolated from breast ductal carcinoma in situ (Sang et al. 2008). In this sense of switching cellular proliferation states, one could construe observed less synchronised proliferation properties of G38 and G40 TiCs when compared to matched differentiated tumour cells (Figure 5).

However, the mechanisms underlying the process of dormancy and re-entering the cell cycle accompanied by the capability of tumour initiation and invasive growth are far from fully understood (Patel and Chen 2012). As proliferation and ‘stemness’ are believed to be maintained in a large part by reciprocal signalings in tumour microenvironment (Hide et al. 2013), data gathered from mere cell-culturing experiments cannot capture the conditions found within a tumour-containing tissue. As outlined in the introduction, perivascular and hypoxic niches represent appropriate sites, where CSCs may reside. Thus, hypoxia was recently documented to directly contribute to dormancy of GCSCs. By inducing protein phosphatase 2A (PP2A) expression, hypoxic conditions were shown to mediate G₁/S phase growth inhibition and reduced cellular ATP consumption in GCSCs. Otherwise, inhibition of PP2A activity resulted in increased cell proliferation, ATP exhaustion and accelerated p53-independent cell death of hypoxic TiCs (Hofstetter et al. 2012).

Furthermore, secretion of growth factors by surrounding astrocytes, oligodendrocytes and microglia has frequently been related to growth characteristics of glioma cells (Du and Dreyfus 2002, Hoelzinger et al. 2007). EGFR gene amplification, rearrangements and overexpression are particularly conspicuous features of glioblastoma. Genes induced by EGFRvIII, a characteristic EGFR mutant expressed in glioblastoma, include the wild-type EGFR ligands transforming growth factor-α (TGF-α) and heparin-binding EGF (HB-EGF) indicating that EGFRvIII plays a causal role in generating an autocrine loop that contributes to a growth autonomy of glioblastoma cells (Ramnarain et al. 2006). Li et al. even obtained neurosphere-like colonies exhibiting immunoreactivity for CD133 and nestin, when GCSCs were cultured in pure DMEM/F-12 medium lacking any additional growth factor substrates. Sphere-forming assays revealed the capacity of dissociated primary tumour spheres to yield secondary tumour spheres concomitant with their ability to self-
renewal and proliferation. As EGF and bFGF mRNA were amplified in glioblastoma sub-
spheres as well as Western blot analysis confirmed additional EGF and bFGF protein ex-
pression, the authors hypothesised that essential autocrine signals could promote self-
renewal and proliferation of GCSCs in pure DMEM/F-12 medium (Li et al. 2009). With
that said coexistence of a different proliferation state of GCSCs within a single tumour
specimen observed for G38 and G40 tumour samples might even be attained by stem cell
intrinsic properties themselves, since microenviron mental interference was excluded by
choosing cell-culture conditions in abovementioned study of Li and colleagues.
Anyhow, it is a debatable point to what extend unsynchronised growth of GCSCs might
represent an indispensible characteristic of overall glioblastoma tumour malignity, as it
was not shown that markedly in case of G35 TiCs (Figure 8A) in our study. However, rap-
idly-cycling CSCs might not be present all along, but rather emerge in various periods of
time. Further investigation might clarify whether such intrinsic unsynchronised growth
patterns of CSCs could vary over time in a way that a whole CSC population might occupy
a slowly-dividing state.

4.3. Examination of DNA-fragmentation of stem-like glioblastoma cells
in the context of a putative resistance to temozolomide and radia-
tion

The quiescent nature of cancer stem cell pools has often been related to conceivable inher-
ent resistance and therefore cell survival to conventional therapies, as radio- and chem-
otherapy are said to predominantly target rapid-cycling cells or at least require active cy-
cling for induction of apoptosis. For example, in a study of Gao and colleagues, ovarian
CSCs exhibited a greater cisplatin $IC_{50}$ value than differentiated tumour cells indicating
that the CSC fraction was selectively resistant to the chemotherapeutic agent cisplatin
compared to the none-CSC population (Gao et al. 2010). CSCs that survive standard treat-
ment strategies might be able to re-enter the cell cycle, give rise to highly-proliferating
posterity and re-establish the tumour bulk after therapy-induced initial shrinkage of the
malignant mass (Moore and Lyle 2011). As tumour recurrence after surgical removement
and radiochemotherapy has been described a common characteristic of glioblastoma (Giese
et al. 2003, Wong et al. 1999), we next aimed at contrasting DNA-fragmentation as an in-
dication of cell death of TiCs and differentiated glioblastoma cells in response to standard
treatment modalities. Despite a substantial amount of barely proliferating TiCs within all
three examined GCSC populations, our findings on DNA-fragmentation rates of treated glioblastoma cells measured 120 h after irradiation or administration of temozolomide did not point to the idea of a less percentage of cells undergoing cell death in response to radiochemotherapy to represent a distinguishing characteristic of TiCs and differentiated glioblastoma cells. However, these observations ought to get in line in a highly controversial debate on potential GCSC resistance. Eramo et al. first evaluated the chemosensivity of GCSCs applying serum-free medium supplemented with EGF und bFGF, which is to say under conditions optimised for culturing of free-floating suspension cells. Temozolomide was scarcely effective in induction of apoptosis in glioma progenitors and TiCs were able to recover and proliferate, although at lower levels than untreated cells (Eramo et al. 2006). CD133+ GCSCs exhibited a significantly lower decrease in the percentage of viable cells than CD133– and nestin-negative glioblastoma cells when treated with up to 2000 µM of temozolomide for 48 h. Even the highest TMZ concentration was not able to trigger a cell death in more than about 40 % of TiCs, whereas even more than 50 % was detected for differentiated tumour cells (Liu et al. 2006). As opposed to this, Beier and colleagues described that temozolomide preferentially depleted GCSCs coming along with a reduced number of equally sized tumour spheres, while Gds were resistant to the agent (Beier et al. 2008). Recent data have also questioned the frequently quoted study of Hegi et al. that suggested MGMT promoter methylation to render glioblastoma more sensitive to temozolomide as already described in the introductory part (Hegi et al. 2005). Blough and colleagues were not able to demonstrate a statistically significant association between sensitivity to TMZ and MGMT methylation in GCSCs isolated from 20 patients glioblastoma. However, they found a significant association between response to TMZ and the expression of MGMT transcript and protein. Therefore, rather transcript and protein expression than methylation status of MGMT might be indicators of a possible benefit from TMZ treatment. Interestingly, the authors could not see any association of GCSCs in response to temozolomide (Blough et al. 2010). Thus and even against the backdrop of a known positive MGMT expression, but an unknown MGMT promoter methylation status, we could suppose that - compared to adherent cells and seen over a period of five days after treatment - the GCSCs used in our study obviously did not feature any more pronounced intrinsic mechanisms to counter treatment with temozolomide by a less percentage of cells exhibiting distinct DNA-fragmentation as an indication of cell death. However, seen over this relatively brief span, conclusions on a putative intrinsic resistance of GCSCs in its entirety are hardly to draw. Instead, it is important to also point to publications that suggested
glioblastoma cells to mainly respond to TMZ by G$_2$-M arrest, but scarcely by increased levels of DNA-fragmentation. According to this, treated p53-proficient U87 glioblastoma cells were reported to accumulate at the G$_2$-M boundary two days after administration of the chemotherapeutic agent. This G$_2$-M arrest was maintained for at least ten days and the sub-G$_1$ population that was equivalent to the amount of DNA-fragmentation measured in our settings did not significantly increase throughout the ten days after TMZ treatment. In contrast, a gradual increase in cells within the sub-G$_1$ was observed when p53-deficient glioblastoma cells were exposed to TMZ. Moreover, G$_2$-M arrest began to decrease by three days after treatment and was substantially lessened until day ten (Hirose et al. 2001). Therefore, further experiments might clarify whether the observed sub-G$_1$ population would increase for extended periods of time compared to measurement 120 h after treatment in our work, as initially G$_2$-M arrested cells might transition into the sub-G$_1$ peak. Seen over this time, exceeding G2-phase arrest of CSCs coupled with putative increased DNA repair protein activity might unveil a survival advantage over bulk cells, which in turn might ultimately be forced to undergo apoptosis (Moore and Lyle 2011). As nowadays the microenvironmental impact on drug resistance of hematological malignancies as well as of many solid tumours is considered an undoubted fact (Meads et al. 2008), it might also markedly contribute to chemoresistance in glioblastoma. So far, three different mechanisms how chemoresistance might be mediated by the surrounding tissue have been described: direct cell-cell interactions, local secretion of cytokines like II-6 and micro-environmental conditions, such as hypoxia (Beier et al. 2011). However, there are only indirect hints that could explain different effects of chemotherapy on GCSCs and Gds. Transcription factor STAT3 has been shown to increase the resistance of GCSCs to TMZ (Villalva et al. 2010, Beier et al. 2011). As II-6 produced by the surrounding brain tissue ranks among various cytokines that can activate production of STAT3, it might selectively contribute to chemoresistance of cancer stem cells. Thus, data obtained from in vitro experiments solely can only provide limited assertions on chemoresistance in its entirety. Similar limitations have to be brought in regarding our experiments on DNA-fragmentation after one-time irradiation. Measured 120 h after treatment again, CSCs did not exhibit a significant lower percentage of cells numbered among the sub-G$_1$ population when compared to matched adherent cells. In 2006, Bao et al. suggested that GCSCs play a decisive role in glioblastoma radioresistance and therefore tumour repopulation after conventional therapies. After ionizing radiation treatment, the fraction of GCSCs increased fourfold relative to untreated cultures portending that CSCs might contribute to cells sur-
4. Discussion

Surviving radiation in a higher percentage than their differentiated progeny do. Albeit radiation-induced DNA damage was detected to similar extent in CSCs and non-CSCs, the former exhibited higher activating phosphorylation of the DNA damage checkpoint proteins ataxia-telangiectasia-mutated (ATM), Rad 17, ChK1 and ChK2. Besides, Rad 17 displayed greater basal phosphorylation in CSCs. The authors postulated that CSCs therefore might be able to occupy a primed state in the sense of a higher basal rate of DNA repair in responding to genotoxic stresses (Bao et al. 2006). Untreated CD133^+ cells have been shown to feature enhanced activation of ChK1 and ChK2 kinases compared to CD133^- cells also suggesting an elongated cell cycle and enhanced basal activation of checkpoint proteins that might contribute to radioresistance. However, as neither DNA base excision nor single-strand break repair nor resolution of pH2AX (the phosphorelated form of histone H2AX) were increased in GCSCs, enhanced DNA repair was not believed a common feature of these cells (Ropolo et al. 2009). With such data on hand CSCs have commonly been proposed to utilise an unique system of efficient DNA repair resulting in radioresistance, whereby the underlying mechanisms of enhanced DNA repair or a delay in cell cycle progression are still under investigation (Su et al. 2011).

Despite these considerations, our data gathered from three glioblastoma specimens demonstrate that measured five days after treatment GCSCs do not exhibit less numbers of death cells compared to the adherent tumour cells. Provided that CSCs might harbour any intrinsic resistance to radiochemotherapy its effects on cell survival would become manifest not until beyond this period of time.

4.4. Glioblastoma TiCs and ds revealed differing protein detection levels of several apoptosis-mediating key players

Several molecular signalling pathways are believed to play an essential role in maintaining stemness, immortal growth, tumourigenic capacity and anti-apoptotic features of GCSCs (Yamada et al. 2011). Therefore, we focused on several key regulatory proteins that have been reported to be expressed in different levels in GCSCs and matched differentiated counterparts and analysed whether these players might also be present in a variable extent in the glioblastoma TiCs and ds used in this work. First, we thereby examined protein expression levels of several BCL-2 family proteins that - as highlighted in the introductory part - have been shown to play a crucial role in governing commitment to apoptosis according to the intrinsic pathway (Abdullah and Chow 2013). While pro-survival BCL-2 family players exhibited greater protein expression in all three adherent cell populations, a
predominant protein expression of the pro-apoptotic BCL-2 repertoire was detected in GCSCs. In addition to a prevalent mitochondrial localisation, immunofluorescence staining of selected BCL-2 family members could demonstrate that these disparities detected by Western blot analysis were also present on a single-cell level and could not be ascribed to overexpression in any subpopulation within the six used primary cell populations, respectively. Furthermore, in case of matched primary cell lines G38, RT-PCR of one selected proapoptotic BCL-2 protein revealed differences in mRNA levels to be at least partly responsible for observed disparities on protein expression level. However, as this was not the case for G35 TiCs and ds, further experiments using e.g. proteasome-inhibitors or anti-ubiquitin antibodies might clarify whether disparities in protein degradation contribute to detected differences of intracellular protein amount.

As opposed to our data, a vast majority of publications demonstrated what appeared to be quite opposite results. For example, Konopleva et al. reported that the anti-apoptotic proteins BCL-xL and BCL-2 were overexpressed in quiescent CD34⁺ AML progenitor cells. Upon differentiation initiated by treatment with all-trans retinoic acid, expression of these proteins could be reduced coming along with enhanced chemosensitivity to cytosine arabinoside suggesting a chemoresistance that was at least partly driven by BCL-2 overexpression (Konopleva et al. 2002). Similarly, CD44⁺ breast cancer stem cells as well as chemoresistant CD133⁺ human hepatocellular carcinoma cancer stem cells have been shown to be associated with high levels of BCL-2 expression (Madjd et al. 2009, Ma et al. 2007). In contrast to abovementioned publications linking BCL-2 overexpression to a survival advantage of CSCs upon chemoradiotherapy, our results offer the idea of glioblastoma TiCs that - especially with regard to additional overexpression of caspase-3 compared against glioblastoma ds - might even feature a primed state towards apoptosis. However, this state does not inevitably have to entail an irreversible phase leading to cell death, as DNA-fragmentation of both untreated glioblastoma TiCs and ds was approximately detected at a 10% - level. In additional analysis of about equal DNA-fragmentation rates in response to temozolomide treatment or irradiation, one could hypothesise that - seen over a time exceeding that of 120 h after treatment - the GCSCs in our setting might indeed not reveal any more pronounced intrinsic resistance towards radiochemotherapy as against differentiated descendants. However, lacking further experiments examining DNA-fragmentation and cell viability for extended periods of time, this assertion is not beyond the scope of assumptions.
4. Discussion

Besides, observed differing protein expression profiles of several key proteins involved in EGFR-signalling also portent to the PI3K/AKT and MEK/ERK pathways to be of different significance in the context of cancer stem cell and differentiated tumour cell biology. Notably, concordant protein expression of ERK and pERK that in our study was markedly more enriched in CSCs than matched Gds points to a different weighting of this MAP-kinase, especially with relation to certain publications linking inactivation of MEK/ERK to reduction of sphere formation capacity and differentiation into neuronal and glial lineages (Sunayama et al. 2010, Huse and Holland 2010). Along these lines targeted inactivation of the MEK/ERK signalling inhibited self-renewal and induced differentiation of glioblastoma cancer stem-like cells, since inhibition of sphere-formation of GCSCs was detected even though the inhibitory infect became less pronounced with sequential passage. However, inhibition of both the PI3K/mTOR and MEK/ERK signalling resulted in significantly enhanced differentiation of CSCs accompanied by a reduced ability to form secondary, tertiary and quarterly spheres (Sunayama et al. 2010). Though AKT inhibition has been demonstrated to reduce motility and invasiveness of unselected glioblastoma cells, the impact on cancer stem cell behaviour was significantly greater, since AKT inhibitors more potently reduced the number of viable GCSCs compared to differentiated tumour cells (Eyler et al. 2008).

These results indicate that maintenance of stem cell abilities in glioblastoma might be closely linked to EGF/EGFR signalling and its downstream effector activation (Yamada et al. 2011) suggesting that a combinatorial disruption of the PI3K/mTOR as well as MEK/ERK pathway would represent an effective strategy in the treatment of GCSCs (Sunayama et al. 2010).

Additionally, regarding the examined expression profiles that in a great part markedly differred between TiCs and their differentiated progeny together with DNA-fragmentation rates on day 5 after TMZ treatment or irradiation, one could construe that, although exhibiting about equal percentage of cells that underwent cell death, glioblastoma TiCs and ds might make use of a different weighting of survival pathways to counter chemoradiotherapy. All together, different strategies might be required in view of eradication of both matched cellular entities.
4. Discussion

4.5. Glioblastoma TiCs and ds did not distinctly differ in tumour-initiating capabilities and presented a cell-type plasticity in the sense of a bidirectional interconversion

Despite the deserts gathered from sphere-forming assays as a chosen option to evaluate self-renewal and differentiation of TiCs in vitro, the transplantation assay in immunodeficient mice has yet remained the gold standard of examining the only agreed upon defining feature of CSCs that is to say their ability to initiate tumour development when injected at low cell numbers (Clarke et al. 2006). We therefore finally aimed at contrasting tumour-initiating capabilities of glioblastoma TiCs and ds in the course of animal experiments. As intracranial transplantation would capture more persuasively the conditions glioblastomas were exposed to in a patient’s brain, also indicated in a publication of Galli et al., whereupon tumour stem cell lines reproducibly established tumours with a take efficiency of 100 % orthotopically, but only of 50 % when injected subcutaneously (Galli et al. 2004), we decided to make use of an orthotopic animal model in our settings.

Even the low multiplicity of about 500 G35 ds has shown to generate a marked tumorous mass, although the quantity of implanted cells was far below the reported numbers of differentiated tumour cells that nonetheless failed to form a tumour in mice (e.g. 500,000 CD34+ CD38+ AML-cells (Lapidot et al. 1994), 20,000 CD24+ breast cancer cells (Al-Hajj et al. 2003), 100,000 CD44– CD24– ESA– pancreatic tumour cells (Li et al. 2007). As the cell populations in this study, termed TiCs and ds, were characterised by an enrichment in stem cell marker nestin and differentiation marker GFAP, respectively, but not a distinct purity, the possibility of cellular contamination in terms of admixture of nestin-positive tumour cells to adherent differentiated cell populations certainly could not be excluded. Anyway, if glioblastoma only originated from stem cell marker-positive tumour cells, longer survival of mice would be expected after injection of Gds compared to TiCs. However, regarding Figure 14, this was not confirmed in our experiments. Furthermore, in case of injection of 500 Gds, tumour formation would be in particular unlikely, as a putative amount of stem cell marker-positive cells among the differentiated cell populations certainly ranked below the lowest reported number of about 100 CSCs that were able to induce tumour formation (Singh et al. 2004). Therefore, the observed and comparably pronounced capability of GCSCs and Gds to initiate tumour formation in immunosuppressed mice consequently raises doubts about tumour initiation originated from low transplanted cell numbers to represent a distinguishing feature of CSCs and their progeny in case of the
malignancy of glioblastoma in general or at least in case of the three glioblastoma specimens, isolated cells of which were taken as a basis of ensuing experiments in this study. However, interpreting our results obtained from transplantation experiments, it has to be mentioned restrictively that transplantation assays in the sense originally presented by Bonnet and Dick in 1997 comprise a serial character, i.e. CSCs, now isolated from the rodent’s tumorous mass, have to prove themselves able to form tumours again that recapitulate the diversity of the primary lesion found in the patient. Even though this concept of ‘serial’ transplantation was not complied in the present thesis, observed tumour formation initiated from about 500 implanted, mainly GFAP-positive adherent glioblastoma cells did not fit with the generic view tumour initiation to only be started from low numbers of CSCs. Similar observations were described in case of malignant melanoma, whereupon the use of a highly immunocompromised mouse strain increased the detection of tumorigenic cells from 1 in 837,000 estimated in NOD/SCID mice to 1 in 4 unselected melanoma cells for NOD/SCID-II2Rγ-null rodents (Quintana et al. 2008), therefore questioning whether tumour-initiating capabilities would indeed pertain to only a small subset of commonly termed CSCs or TiCs. Hence, one could suppose that tumorigenic cells might actually be very common in glioblastoma and the potential of tumour initiation might not only be occupied by nestin+/GFAP− cells growing in spheres in vitro. As a consequence, the term of TiC could also find a use in the context of differentiated adherent glioblastoma cells, therefore essentially modifying unless challenging the CSC-hypothesis for this malignancy.

However, it is important to recognise that for want of alternative and more suitable methodical approach these considerations are underlain by attempts to identify CSCs solely by tumorigenicity assays for sorted cancer cell subpopulations (Grotenhuis et al. 2012) and staining for conjectural, but - as stated above - partly doubtful stem cell/differentiation markers. The interpretation of such xenotransplantation studies is complicated by the critical role in tumour growth concerning interactions with the microenvironment that might lead to the survival of those implanted cells that could rapidly adapt to growth in a foreign animal milieu (Kelly et al. 2007), but not necessarily of those that drive tumour development in the donor’s setting.

Referring to the abovementioned study of Quintana and colleagues, William Kaelin from Dana-Farber Cancer Institute in Boston, Massachusetts, who however was not affiliated with this publication, nevertheless commented that, if it turned out that the cells [i.e. CSCs] had never been rare, then we had been studying those cells all along [...] and the cancer stem cell was just a cancer cell (cited in Baker 2008). However, in our case of glioblas-
4. Discussion
toma, the distinct and differing protein expression profiles of untreated GCSCs and their differentiated counterparts shown in Figure 10 seem to refute this statement. It rather appears that the question for the concept of CSCs has to be assessed depending on the respective malignancy in the context of which it is proposed. Thus, the evidence for CSCs in several cancers especially those of the hematopoietic system, where a pool of hematopoietic stem cells is responsible for a lifelong replenishment of major blood cells, is particularly convincing. Accumulating knowledge suggests that the acquired BCR-ABL mutation of the Philadelphia chromosome leading to a constitutive activation of tyrosine kinases as outlined in the introductory part initiates chronic phase CML and results in aberrant stem cell differentiation and survival. As a consequence, an expanded progenitor population is produced that acquires self-renewal capacity resulting in leukemia stem cell generation and blast crisis transformation (Jamieson 2008). By way of contrast, glioblastoma exhibiting strongly pronounced chromosomal instability, polyploidy as well as inter- and intratumor-uous heterogeneity may constitute the other extremity, where the origin of putative CSCs is not yet known and GCSCs might emerge from an already existing tumorous mass through accidental dedifferentiation of differentiated glioblastoma cells. In this sense one could construe our findings of comparably low numbers of occasional nestin-positive glioblastoma cells irrespective of tumour formation initiated by transplantation of cell populations markedly enriched in nestin-negative or nestin-positive glioblastoma cells in a NOD/SCID-II2Rγ-null mouse model (Figure 16). However, in order to counter a possible unspecific or artifactual binding of nestin antibody, data should be validated by staining of further proteins, such as SOX2, said to be essential for maintaining self-renewal of putative CSCs. Despite we did not go into this limitation, our data point to a cell-type plasticity that might cause a bidirectional interconversion between nestin-positive CSCs and differentiated glioblastoma cells. While dedifferentiation of nonstem cancer cells leading to the generation of cancer stem cells has already been reported for several other malignancies, such as colon cancer (Su et al. 2011) and prostate cancer (Jeter et al. 2011) among others, Friedmann-Morvinsky and colleagues have succeeded in yielding nestin and SOX2 positive cancer stem cells out of genetically altered astrocytes in case of glioblastoma, yet. Therefore, they have used a mouse glioma model based on two short hairpin RNAs to silence the genes encoding neurofibromatosis type 1 (NF1) as well as tumour suppressor p53 (Friedmann-Morvinski et al. 2012). Consequently, the authors could observe induction of tumours that rapidly progressed towards an invasive phenotype. However, notwithstanding histological analysis of dissected tumours revealed characteristics commonly found within
human glioblastoma specimens, such as increased cellularity, vascularity, hemorrhage and necrotic areas, it is arguable whether silencing of two genes that are implicated in malignant transformation processes in a plethora of tumours is sufficient to capture genotypical characteristics of glioblastoma, particularly as mutations of NF1 and p53 can be detected in 18% respectively 35% of primary glioblastoma (Friedmann-Morvinski et al. 2012), but certainly do not rank among the most frequently mutated proteins of this malignancy (see introduction). We therefore decided to examine cell characteristics of ‘human’ glioblastoma cells in a NOD/SCID-II2Rγ-null mouse model that compared to the NOD/SCID mice was characterised by not only a lack of B- and T-cell activity, but also of natural killer (NK) cells, which are thought to be responsible for the rejection of most transplanted human cells (McKenzie et al. 2005). The objection of a missing immune system that certainly would affect tumour biology in a patient has to be acquiesced admittedly. However, application of a mouse model that admits the presence of an unsuppressed murine immune system seems not to refute this limitation, but might be even more incommensurable to a human environmental setting, as it has been reported that human genomic responses to several examined stresses, such as trauma, burns and endotoxemia, are not reproduced in the current mouse models (Seok et al. 2013).

Considering the animal experiments presented in this study, we can suppose that glioblastoma cancer stem cells characterised by the relative expression of stem cell marker nestin are likely not to be required for tumour initiation of glioblastoma, as even transplantation of GFAP+/nestin– adherent tumour cells yielded in formation of a tumorous mass. This consequently raises the question whether the widely used synonym tumour-initiating cell can live up to the term cancer stem cell. It rather seems that the CSC would not comprise an entity that is indispensible for glioblastoma initiation and formation, but might contribute to tumour progression and recurrence of glioblastoma after treatment.

Making use of primary human glioblastoma cell lines, our results additionally propose a rather bi- than unidirectional interconversion between GCSCs and nonstem cells, where-upon differentiated tumour cells may also occupy the ability to dedifferentiate into a more stem-like state. Cell-type plasticity seems to constitute an imperative feature of glioblastoma.
5. Summary

Geared to further investigate whether some of the frequently postulated perceptions concerning the so-called concept of cancer stem cells would also be valid in case of glioblastoma, this study was underlain by comparative experiments on matched pairs of glioblastoma cancer stem cells (GCSCs) and their adherent, differentiated progeny (Gds). Thereby, putative free-floating GCSCs isolated from three human tumour specimens predominantly exhibited positive staining for the stem cell marker nestin as well as - after switching culture conditions towards differentiation - adherent nestin-negative tumour cells occurred that proved to mainly express the mature astrocyte marker glial fibrillary acidic protein (GFAP).

In keeping with the idea of quiescent and slow-cycling CSCs, all three GCSC populations have been shown to contain a large percentage of barely proliferating cells, whereas within two of them a marked unsynchronised growth pattern revealed a highly dividing subpopulation that did not lag behind the quantity of cell divisions of differentiated counterparts. Despite observed disparities of proliferation that are commonly regarded as key to the efficacy of radiochemotherapy which mainly targets rapidly-cycling cells, GCSCs did not feature lower DNA-fragmentation rates in response to administration of temozolomide or exposure to radiation when measured 120 h after treatment. Having found no clear differences in cell death sensitivity, we therefore investigated the expression of key mediators of treatment-induced apoptosis. Among others protein expression of pro-apoptotic B-cell-lymphoma-2 (BCL-2) family players detected for GCSCs clearly surpassed that of adherent glioblastoma cells. Inverse findings were present in case of anti-apoptotic family members. Taken together these data suggest that, while sensitivity towards apoptotic stimuli was not fundamentally different between GCSCs and Gds, the cell populations seemed to follow different survival strategies on a molecular level.

Subsequently, tumour-initiating capabilities of one matched pair of glioblastoma cells were analysed in an orthotopic mouse model revealing obvious tumour formation for both injection of conjectural GCSCs and differentiated glioblastoma cells even when transplanted at low cellular multiplicities. As tumour formation initiated by a less number of tumour cells was considered the most generally accepted distinguishing feature of CSCs and the remaining tumour cells, our results obtained from transplantation assays seemed to harbour doubts on this view in the context of glioblastoma. Finally, staining for the stem cell marker nestin revealed positive cells even in cases of tumours initiated by transplantation of adherent GFAP-positive tumour cells. These findings suggest the idea of a cell-type
plasticity in the sense of a bidirectional interconversion between GCSCs and non-stem tumour cells, whereupon differentiated tumour cells might undergo dedifferentiation to generate a more progenitor (cancer stem cell) state.
6. **Bibliography**


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7. List of Figures

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Statutory declaration

Hereby, I declare that I wrote the present dissertation with the topic:

“A paired comparison between glioblastoma cancer stem cells and differentiated cells in view of proliferation, resistance to conventional therapies and tumour-initiating capabilities”

independently and used no other aids that those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works.

Furthermore, I hereby declare that I have carried out my scientific work according to the principles of good scientific practice in accordance with the current “Satzung der Universität Ulm zur Sicherung guter wissenschaftlicher Praxis” [Rules of the Ulm University for Assuring Good Scientific Practice].

Ulm, 02.03.2015

Matthias Schneider
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Not to forget: my PC deserves great thanks, too, for having crashed never once throughout working on the written part of this thesis.
# Curriculum vitae

## Personal dates

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## Educational training

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## Junior studies (school accompanying)

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<th>Details</th>
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<tr>
<td>2006 - 2011</td>
<td>State University of Music and the Performing Arts Stuttgart</td>
<td>Artistic Education in piano, class of Prof. S. Rudiakov</td>
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<tr>
<td>07/2011</td>
<td>Graduation: Diploma Artistic Education in piano</td>
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<tr>
<td>2011 - 2012</td>
<td>Continuation of piano education, class of Prof. S. Rudiakov</td>
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<tr>
<td>Since 2013</td>
<td>University of Music Würzburg</td>
<td>Continuation of piano education, class of Prof. B. Glemser</td>
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<tr>
<td>2007 - 2014</td>
<td>University of Ulm</td>
<td>Study of human medicine</td>
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<tr>
<td>11/2014</td>
<td>Graduation: License as a physician</td>
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Curriculum vitae

Since 2011   Department of Neurosurgery, University Hospital Ulm, (Head of laboratory: Prof. Dr. M.-E. Halatsch) in cooperation with Department of Pediatrics and Adolescent Medicine, University Hospital Ulm (Head of laboratory: Prof. Dr. K-M. Debatin)
Medical doctoral candidate

Occupational development
Since 03/2015   Department of Neurosurgery, University Hospital Bonn
Assistant physician
Publications

