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Three-Dimensional In Vitro Models in Glioma Research – Focus on Spheroids

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1. Introduction

In the field of glioma research, in vitro models are widely used to investigate tumor biology as well as tumor response to chemotherapy and radiation. There is an increasing need to improve these in vitro models in order to meet the new challenges arising in drug discovery. It is thus important that development of new drugs is based on the latest knowledge about glioma biology such as for example the recent discovery of tumor stem cells (Reya et al., 2001). When investigating glioblastomas in vitro – and especially the supposed tumor stem cells – three dimensional multicellular spheroid models have recently come into focus.

The aim of this chapter is to review the development as well as the most recent aspects of the three-dimensional glioma in vitro models focusing on glioma spheroids. The implementation of these models in current and in future in vitro glioma research will be discussed putting emphasis on the themes described below.

Cell lines cultured as monolayers have been the in vitro model of choice for many years (Ponten & Macintyre, 1968). However, the three-dimensional aspect came into focus in the 1970's, where scientists started to grow tumor cells from cell lines as multicellular spheroids (Yuhas et al., 1977). Over the years the spheroid model has been improved by deriving spheroids from cells obtained from dissociated primary glioblastoma tissue (Mackillop et al., 1985) as well as by using organotypic primary spheroids derived from small tumor fragments (Bjerkvig et al., 1990).

In general, most in vitro studies are performed with cells cultured in conventional serum-containing medium. Recently – as the tumor stem cell theory has evolved – the culturing medium has come into focus. It has thus been demonstrated that the use of serum-free medium for culturing of cell line-derived spheroids preserved the in vivo-like features as well as the tumor stem cell-like phenotype suggesting crucial importance of the use of serum-free medium in tumor stem cell research (Lee et al., 2006).

Identification of the glioma stem cells is still a matter of discussion. The most used marker in the field has been the cell surface marker CD133. Expression of this putative tumor stem cell marker in gliomas has been studied in several papers demonstrating clusters or niches of CD133 positive tumor cells as well as CD133 positive single cells dispersed in the tumor

(Christensen et al., 2008; Hermansen et al., 2011). These important tumor stem cell niches are preserved in primary glioma spheroids in contrast to cell line spheroids (Christensen et al., 2010).

Another important aspect in culturing of glioma stem cells is hypoxia, since several studies have shown that hypoxia influences radiation resistance. This may be explained by effects of hypoxia in vitro on proliferation of the tumor cells, spheroid formation and expression of stem cell markers, suggesting that also this aspect should be taken into consideration (Heddleston et al., 2009; Kolenda et al., 2010; McCord et al., 2009; Soeda et al., 2009).

Several studies have used the different types of spheroids mentioned above for investigating the effects of chemotherapy and radiation on the tumor cells and in particular on the tumor stem-like cells (Bao et al., 2006; Bauman et al., 1999; Fehlauer et al., 2005; Fehlauer et al., 2006; Fehlauer et al., 2007; Genc et al., 2004; Gliemroth et al., 2003; Haas-Kogan et al., 1996; Johannessen et al., 2009; Kaaijk et al., 1997; Khaitan et al., 2009; Sunayama et al., 2010; Terzis et al., 1997; Terzis et al., 1998; Wakimoto et al., 2009; Wang et al., 2010). After such treatments cell viability and cell proliferation assays as well as secondary spheroid formation assays have been used to evaluate induced effects. Moreover, expression of apoptosis and proliferation markers and for example stem cell markers have been investigated immunohistochemically in paraffin embedded spheroids (Christensen et al., 2010). The advantages using this panel of methods will be an important part of this chapter.

High grade gliomas are known to be highly invasive and new knowledge concerning tumor cell invasion also incorporating the tumor stem cell aspect is urgently needed. In our laboratory we have worked to improve in vitro models when investigating the invasive features of gliomas. This led to establishment of an in vivo-like model of invasion, where spheroids are implanted into organotypic brain slice cultures.

Taken together the three-dimensional multicellular spheroid model is the three dimensional model of choice for in vivo-like glioma in vitro studies. However, at the same time this model is under ongoing development to become an even more in vivo-like model in order to meet the new challenges of glioma research and drug development. The use of spheroids in especially tumor stem cell research has been fast increasing in recent years making spheroids an important tool also in future glioma research.

2. Establishment and development of the spheroid model

In the field of cancer research it is important to continuously develop in vitro models mimicking in vivo conditions as much as possible. By isolating cells from tumor tissue, tumor cell lines can be established from almost any kind of tumor including brain tumors such as glioblastomas. Glioblastoma cell lines are traditionally cultured as adherent monolayers but can also be cultured as single cell suspensions or spheroids. One of the most used cell lines in glioblastoma research is the cell line U87MG established by Pontén and Macintyre in 1968 (Ponten & Macintyre, 1968). This cell line was established from an astrocytic tumor with necrosis, which corresponds to this tumor being a glioblastoma multiforme according to WHO (World Health Organization) guidelines 2007 (Louis et al. 2007). U87MG was originally established in a traditional serum containing medium and the cells were described as large, extremely bizarre and very slowly growing. Today U87MG can be described as having only limited pleomorphism as well as being fast growing, clearly indicating that such cell lines change over time. U87MG has been used in a variety of glioma studies and is still used. Since the cell line has been cultured in different laboratories for

over 40 years new genomic mutations have arisen capable of giving rise to different phenotypic subpopulations in different laboratories. It is also well known that the phenotypic characteristics and genetic aberrations found within in vitro cells passaged repeatedly for about 10 times in serum containing medium often show only little resemblance with the original primary tumor (Lee et al., 2006). It is therefore not surprising that the U87MG cell line is known to have a highly aberrant genomic structure as visualized by karyotyping (Galli et al., 2004). Galli et al. (Galli et al., 2004) demonstrated loss of chromosome 1, 9, 10, 11, 12, 13, 14, 16, 19, 20, 22 and X. Furthermore, 11 unidentified abnormal chromosomes were found, whereas no gains of chromosomes were seen. In addition to this, Clark et al (Clark et al., 2010) have sequenced the genome of U87MG in order to further characterize it. They identified 35 interchromosomal translocation events, 1,315 structural variations (>100 bp), 191,743 small (< 21 bp) insertions and deletions as well as 2,384,470 single nucleotide variations. Protein coding sequences were disrupted predominantly by small insertions and deletions as well as larger deletions and translocations and 512 genes were homozygously mutated. Surprisingly, the study by Clark et al. also indicated that although this U87MG cell line has been cultured for more than 40 years, the cell line has now been relatively stable for years and is not rapidly changing anymore. This relative stability could be an advantage when using the U87MG cell line. In addition, the use of this cell line for four decades has resulted in a very well characterized cell line.

In general, there are many advantages when using cell lines. When first established, they are easy to handle in the laboratory and a large number of cells can be obtained in a short period of time, making it feasible to conduct large scale studies. In addition, cell lines are relatively easy to manipulate genetically by transfection and knock-down etc. establishing subpopulations with specific gene expression. These cell lines are important tools in the field of basic research investigating cellular pathways involved in tumor biology and response to different drugs. In addition to the obvious advantages, there will always be challenges when working with cell lines. As mentioned above, tumor cell lines are very likely to acquire new mutations and chromosome damage when undergoing cell division, because of the unstable genome in the tumor cells. The longer cultures are maintained and passaged the more changes accumulate (Lee et al., 2006) leading to changes in tumor cell behaviour. This is one of the main problems by culturing cell lines for many years. There will always be a possibility that the cells further mutate and several subpopulations will arise. This is important to keep in mind, when comparing results obtained with the same commercial cell line but in different laboratories at different time points.

Another obstacle to overcome when using cell lines is the heterogeneity seen in tumors like glioblastomas. It is not possible to maintain the high degree of heterogeneity in long term cell cultures. In order to improve models using cell lines, short term cultures prepared from fresh tumor biopsies can be an alternative (Kolenda et al., 2010; Potter et al., 2009). The use of short term cultures may reduce differences between the tumor of origin and the cultured cells. In a study by Potter et al. (Potter et al., 2009) short term cultures from 6 pediatric pilocytic astrocytomas and 3 adult glioblastomas were established and cultured in conventional medium containing fetal calf serum. Gene expression profiles of the derived short term cell cultures harvested below passage 8 and their respective original biopsies were performed. They demonstrated that although short term cultures more resemble in situ gliomas than homogenous long term cultures, significant changes in gene expression were found between the biopsies and the derived short term cultures. The most significant

functions differing for the glioblastomas were associated with cell structure, shape, motility, proliferation, cellular development, cell death, cellular assembly and organization, cell-to-cell signaling and interaction, as well as cell cycle.

As our knowledge of tumor cells expands, there is a need to establish more advanced models to mimic the tumor in situ. One of the main problems is the fact, that the cells are removed from their natural environment, dissociated and cultured as single cells. It may therefore be important to prevent this in order to be able to mimic the natural environment as much as possible. Moreover in the recent years, the use of serum containing cell culturing medium has come into focus. The composition of serum has not been fully understood for many years, but it is known that it supplies the cells with nutrients, vitamins, hormones, and growth-, differentiation-, and attachment-factors. These factors may affect the cells in ways we are not fully aware of. It is also well known that there are differences between batches of serum (Fisher & Wieser, 1983). Another important issue is the fact that only a small fraction of cells in the organism is in direct contact with serum. This may be a problem in glioma research, since the brain and brain tumor tissues are not among these cells. In this context, it is also worth highlighting that neural stem cells should be cultured under serum-free conditions similar to what has been found for the so called glioblastoma tumor stem cells.

The three-dimensional glioma spheroid model came into focus in the 1970's, where scientists started to grow tumor cells as multicellular spheroids using tumor cells from conventional monolayer cultures (Yuhas et al., 1977). Such spheroids are usually formed by aggregation of cells growing into the larger three-dimensional spheroids. They are believed to be a better model than monolayer cultures due to a three-dimensional structure with more in vivo-like intercellular contacts. This model was later on further improved by deriving spheroids from single cells obtained from dissociated primary glioblastoma tissue (Mackillop et al., 1985). In order to obtain an even more in vivo-like model the primary organotypic spheroids were introduced (Bjerkvig et al., 1990). Organotypic means that the properties characteristic of the tissue of origin is maintained. These spheroids are derived from freshly removed glioma tissue and have been shown to be a valid tumor model providing a biological system that mimics the original glioma in vivo.

When deriving primary spheroids from glioma biopsies, it is important to process the tumor tissue as soon as it is removed. As we have published earlier (Christensen et al., 2010) the glioma tissue should be collected directly in the operation theatre, where the tissue is placed in a tube with Hanks' Balanced Salt Solution supplemented with 0.9 % glucose and transported to the laboratory. The tumor tissue can then be processed according to the study by Bjerkvig et al. (Bjerkvig et al., 1990), where small tumor fragments of approximately 200-400 µm in diameter are obtained after sectioning the tumor tissue manually using scalpels. These fragments are then transferred to 0.75% agar-coated culture flasks of 75 cm² with pre-warmed medium. The cultures should be kept in a standard tissue culture incubator (95% humidity, 95% air, and 5% CO_2) and the following day the culturing medium should be changed in order to remove dead blood cells and cellular debris. The tumor fragments should then be examined under a light microscope every day, until they round up to form spheroids within 5-15 days.

The main advantages by primary organotypic spheroids are the preservation of the original intercellular contacts and the tumor heterogeneity. However, because of this heterogeneity it is important to include a larger number of spheroids in in vitro studies using primary spheroids in order to obtain reproducible results. In a study by Bjerkvig et al., (Bjerkvig et

al., 1990) it was shown that when culturing small tumor fragments from astrocytic brain tumors of increasing grade, small primary spheroids were formed for the majority of the tumors within 3-5 days. The spheroids were analyzed by light microscopy as well as transmission- and scanning electron microscopy (TEM and SEM, respectively) after 3 and 10 weeks of culture, showing the unique preservation of cell- to cell interaction, blood vessels, extracellular matrix, and macrophages. It was moreover demonstrated that the primary spheroids could be cultured for 70 days with preservation of the histology of the spheroids. In a similar study in our laboratory, glioma tissue was collected from 11 patients. The tissue pieces from 7 of these patients formed vital spheroids within a week. Thereafter, the spheroids were fixed, paraffin embedded and investigated immunohistochemically as described later in this chapter. Areas of necrosis were seen in some of the spheroids, whereas blood vessels were present in the majority of the glioma-derived spheroids.
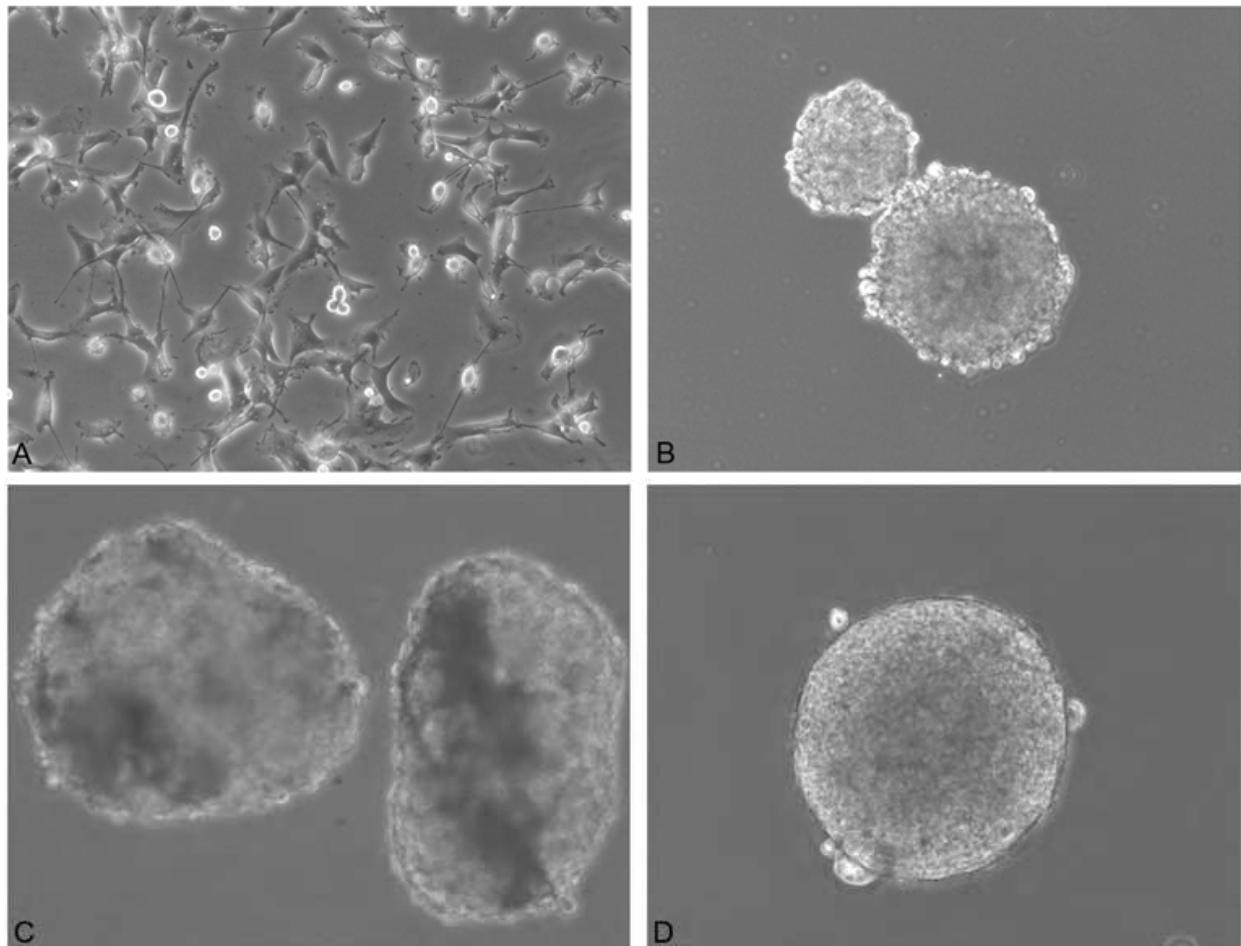

Fig. 1. Adherent monolayer cells and free floating spheroids. U87MG grows as an adherent monolayer when the cells are cultured in serum containing medium (A). However, in serum-free medium U87MG grows as spheroids (B). Tissue derived from freshly removed glioblastoma (C) and cells from a glioblastoma short term culture (D) also grow as spheroids when cultured in a serum-free medium. The organotypic spheroids (C) can be grown in serum containing medium as well and preserves in both media some of the characteristics found in the primary tumor such as tumor necrosis and blood vessels, whereas the short term culture spheroid in (D) has lost these characteristics.

1. Collect tissue and transport the freshly removed tumor tissue in Hanks' Balanced Salt Solution supplemented with 0.9% glucose
2. Place the tissue in a sterile petri dish
3. Section the tumor tissue manually using two scalpels until tumor fragments of 50-400 μm in diameter are obtained
4. Culture fragments in 0.75% agar coated culture flasks containing 20 ml medium
5. Incubate the cultures in 36°C humidified air containing 5% CO_2 and 95% atmospheric air
6. Change the medium the next day
7. Change the medium twice a week in 10-15 days until the fragments round up and form spheroids

Box 1. Preparation of organotypic primary spheroids

3. The tumor stem cell paradigm and the spheroid model

The tumor stem cell paradigm proposes that only a small subset of cells – the so-called tumor stem cells - within the tumor cell population is able to initiate and sustain tumor growth (Ward & Dirks, 2007). These tumor stem cells have been found in a variety of different cancers such as leukaemia (Bonnet & Dick, 1997), colon cancer (Daidone et al., 2004), breast cancer (Al-Hajj et al., 2003) and brain cancer (Singh et al., 2004). With the discovery of the neural stem cells (Reynolds & Weiss, 1992) it also became plausible that brain tumors could be derived from the transformation of neural stem cells or progenitor cells (Singh et al., 2004). The neural stem cells were first isolated by Reynolds and Weiss in 1992 (Reynolds & Weiss, 1992). They found a small population of cells isolated from the adult striatum in mouse brain that were able to proliferate and differentiate. They cultured these cells in a serum-free environment supplemented with the growth factors EGF (epidermal growth factor) and bFGF (basic fibroblast growth factor), and the cells grew as neurospheres. When dissociating the neurospheres and re-plating them as single cells new neurospheres developed. Under these serum-free conditions most differentiating and differentiated cells died, whereas the neural stem cells responded to the growth factors and proliferated to form neurospheres (Vescovi et al., 2006). By applying the same conditions to human glioblastoma cells, it was possible to isolate a population of cells that formed tumorspheres. These cells were capable of differentiation and self-renewal (Galli et al., 2004; Ignatova et al., 2002; Lee et al., 2006; Singh et al., 2003). Furthermore, the cells from the tumorspheres gave rise to tumors resembling the primary tumor when injected into the brains of immunodeficient mice, suggesting that a population of the cells isolated were brain tumor-initiating stem-like cells.

When culturing putative tumor stem cells, spheroid models are often used. Especially the clonogenic neurosphere assay is used for preserving tumor stem-like cells in serum-free medium (Lee et al., 2006). In this assay, primary human brain tumor tissue form spheroids after repeated dissociation into single cells. However, cell-to-cell interactions are interrupted and this might affect experimental results obtained with the tumor stem cell line-derived spheroids. This could be particularly important in tumor stem cell research, since the suggested close relationship between brain tumor stem cells and adjacent endothelial cells (Bao et al., 2006; Calabrese et al., 2007) is lost in these spheroids. In addition, the culturing medium has come into focus when performing studies focusing on tumor stem cells. As

mentioned earlier, normal neural stem cells are cultured under serum-free conditions, since it is well known that serum causes irreversible differentiation of neural stem cells (Gage et al., 1995). Lee et al. (Lee et al., 2006) cultured glioblastoma short term cultures in a serum-free medium similar to the medium used for culturing neural stem cells in order to preserve and select for tumor stem-like cells. This serum-free medium consisted of neurobasal medium supplemented with EGF and bFGF, because EGF and bFGF earlier seemed to select for tumor stem-like cells by inducing proliferation of multipotent, self-renewing, and expandable tumor stem cells (Galli et al., 2004; Ignatova et al., 2002; Lee et al., 2006). In the study by Lee et al., (Lee et al., 2006) dissociated glioblastoma cells, cultured as short term cultures, formed spheroids expressing putative tumor stem cell markers but when culturing the selected cells in serum containing medium, they irreversibly differentiated into neural and glial cell lineages. Interestingly, this is in line with the irreversible differentiation of neural stem cells under the same conditions (Gage et al., 1995).

In the search for improvement of in vitro models we performed a study in our laboratory (Christensen et al., 2010), where organotypic primary spheroids were cultured in serum-free medium. In terms of the tumor stem cell concept, culturing of these organotypic primary spheroids in serum-free conditions may be closer to the in vivo situation than using tumor stem cell line-derived spheroids, especially regarding studies of radiation and chemo-sensitivity. We investigated the influence of serum-containing medium and serum-free medium on the phenotype of primary glioma spheroids. The aim was to elucidate whether serum-free medium also favors the presence of tumor cells expressing stem cell markers in these spheroids, when investigated immunohistochemically. The results based on seven malignant astrocytomas WHO Grade III–IV, supported the hypothesis that putative brain tumor stem cells are better preserved in serum-free culture medium with EGF and bFGF. When comparing spheroids from both media, we found increased CD133 expression when culturing primary glioma spheroids in serum-free medium compared to serum-containing medium, which is in line with the study by Lee et al. (Lee et al., 2006) using short term cultures. In contrast to Lee, who found a drastic decrease in Sox2, Bmi-1, and Nestin when culturing short term cultures in serum, we only found a slightly decreased expression of Sox2, whereas Bmi-1 and Nestin were equally expressed in both media. This better preservation of stem cell marker expression in serum-containing medium in primary glioma spheroids (Christensen et al., 2010) may be explained by primary spheroids preserving an intact microenvironment, whereas Lee et al. (Lee et al., 2006) repeatedly dissociated the spheroids. Another interesting observation was that primary glioma spheroids cultured in serum-free medium contained more blood vessels than in serum-containing medium, and furthermore, many blood vessels were hyperplastic. The immunohistochemical comparison showed more CD34 and VWF, but less CD31 in serum-free medium compared to serum-containing medium. This increase was accompanied with more CD133 positive cells, thus suggesting that the close relationship between blood vessels and tumor stem-like cells may be better preserved in serum-free medium.

As it is clear from the tumor stem cell research field, markers specific for tumor stem cells are of crucial importance. This has resulted in the development of a great number of antibodies against tumor stem cell-related proteins. Some of the most important markers in the field of brain tumors have been (table 1) CD133 (Bandopadhyay et al. 2010; Bidlingmaier et al., 2008; Christensen et al., 2008; Dell'albani, 2008; Fargeas et al., 2007; Griguer et al., 2008; Jaszai et al., 2007; Mizrak et al., 2008; Pfenninger et al., 2007; Wang et al., 2008; Zeppernick et al., 2008), A2B5 (Balik et al., 2009; Merzak et al., 1994; Ogden et al., 2008; Piepmeier et al.,

1993; Tchoghandjian et al., 2009), Podoplanin (Goodman et al. 2009; Grau et al., 2008; Mishima et al., 2006; Nakamura et al., 2006; Ogasawara et al., 2008; Ordonez, 2006; Shibahara et al., 2006), Nestin (Dahlstrand et al., 1992a; Dahlstrand et al., 1992b; Dell'albani, 2008; Ehrmann et al., 2005; Ma et al., 2008; Maderna et al., 2007; Strojnik et al., 2007; Wan et al., 2011), Mushashi-1 (Kanemura et al., 2001; Ma et al., 2008; Okano et al., 2005; Sakakibara & Okano, 1997; Thon et al., 2010; Toda et al., 2001), Bmi-1 (Bruggeman et al., 2007; Hayry et al., 2008; Park et al., 2004; Zencak et al., 2005) and Sox2 (Gangemi et al., 2009; Ma et al., 2008; Phi et al., 2008) and new upcoming markers such as ID1 (Kamalian et al., 2008; Maw et al., 2009; Nam & Benezra, 2009; Schindl et al., 2001; Schindl et al., 2003; Schoppmann et al., 2003; Tang et al., 2009), NG2 (Brekke et al., 2006; Chekenya et al., 1999; Chekenya et al., 2002a; Chekenya et al., 2002b; Chekenya et al., 2008; Chekenya & Immervoll, 2007; Chekenya & Pilkington, 2002; Joo et al., 2008; Petrovici et al., 2010; Stallcup & Huang, 2008) and CD15 (Capela & Temple, 2002; Capela & Temple, 2006; Read et al., 2009; Ward et al., 2009). Until now, the most widely used marker in brain tumors has been CD133 (Bidlingmaier et al., 2008; Christensen et al., 2008; Dell'albani, 2008; Fargeas et al., 2007; Griguer et al., 2008; Jaszai et al., 2007; Mizrak et al., 2008; Pfenninger et al., 2007; Wang et al., 2008; Zeppernick et al., 2008).

Markers	Short introduction	References
CD133	CD133 is a cell membrane glycoprotein with five transmembrane domains. CD133 has been found in a variety of non-pathogen human tissues including the brain. However, the function remains unknown. It was identified as a marker of hematopoietic stem cells in 1997 and later as a marker of human neural stem cells. Since 2004, CD133 has been widely used for identifying tumor stem cells in brain tumors. However, some results suggest that CD133 is not specific for tumor stem cells. Downregulation of CD133 in glioma cell lines has been suggested to influence migration, spheroid formation and resistance to chemotherapeutics.	Bandopadhyay et al. 2010; Bidlingmaier et al., 2008; Christensen et al., 2008; Dell'albani, 2008; Fargeas et al., 2007; Griguer et al., 2008; Jaszai et al., 2007; Mizrak et al., 2008; Pfenninger et al., 2007; Wang et al., 2008; Zeppernick et al., 2008
A2B5	A2B5 is a cell surface ganglioside found on white matter progenitors of the oligodendrocyte lineage. A2B5 has been found in gliomas in a population of cells, which are distinct from the CD133+ population but have the capacity to initiate tumors. A2B5 might be involved in glioma cell invasion in vitro, probably because of adhesion of the molecule to basement membrane components.	Balik et al., 2009; Merzak et al., 1994; Ogden et al., 2008; Piepmeier et al., 1993; Tchoghandjian et al., 2009
Podoplanin	Podoplanin is a mucin-type transmembrane glycoprotein found in several normal tissues but not in mature astrocytes, oligodendrocytes	Goodman et al. 2009; Grau et al., 2008; Mishima et al., 2006; Nakamura et al., 2006;

Markers	Short introduction	References
	or neurons. A high expression of Podoplanin has been found in high grade astrocytomas. However, no protein has been detected in diffuse astrocytomas or in normal brain tissue. Podoplanin has been suggested to be expressed in human glioma stem cells. This podoplanin positive cell population formed neurospheres in vitro and tumors in vivo. Moreover, these cells showed increased resistance to radiation.	Ogasawara et al., 2008; Ordonez, 2006; Shibahara et al., 2006
Nestin	Nestin is a protein belonging to the class VI of intermediate filaments and it appears after neurulation in the CNS stem cells. In the normal adult brain, nestin is only expressed in the neural stem cells lining the ventricular wall and the central canal. It is believed to be a marker of proliferating and migrating cells. Nestin has been found in several tumor types including gliomas. The expression of nestin may be related to a dedifferentiated tumor stem cell status, enhanced cell motility, invasive potential and increased malignancy.	Dahlstrand et al., 1992a; Dahlstrand et al., 1992b; Dell'albani, 2008; Ehrmann et al., 2005; Ma et al., 2008; Maderna et al., 2007; Strojnik et al., 2007; Wan et al., 2011
Musashi-1	Musashi-1 belongs to a family of evolutionary well conserved neural RNA-binding proteins. Musashi-1 is found in neural stem cells and progenitor cells in the adult human brain and plays important roles in cell fate decision, including the maintenance of the stem cell state, differentiation, and tumorigenesis. Musashi-1 has been found in a variety of tumors including gliomas.	Kanemura et al., 2001; Ma et al., 2008; Okano et al., 2005; Sakakibara & Okano, 1997; Thon et al., 2010; Toda et al., 2001
Bmi-1	Bmi-1 (B lymphoma Mo-MLV insertion region) is a *Polycomb* group transcription repressor, thought to be essential for self-renewal of neural stem cells and maintenance of the stem cell population by preventing premature senescence. Bmi-1 is found mainly around the ventricles in the subventricular zone and *in vitro* in cortical neural stem cells as well as in progenitor cells. Bmi-1 has been found to be highly expressed in human brain tumors including glioblastomas.	Bruggeman et al., 2007; Hayry et al., 2008; Park et al., 2004; Zencak et al., 2005
Sox2	Sox2 (SRY (sex determining region Y)-box 2) is a transcription factor that plays a role in sustaining self-renewal and maintaining	Gangemi et al., 2009; Ma et al., 2008; Phi et al., 2008

Markers	Short introduction	References
	neuronal stem cell fate. It is found in the ventricular and sub-ventricular zone in fetal brains, but only in the ependymal cells in the human adult brain. It has been found to be highly expressed in glioblastoma cells compared to normal human brain and is believed to be involved in proliferation and tumorigenesis.	
ID1	ID1 (inhibitor of DNA binding 1) belongs to a class of transcription factors known as helix-loop-helix (HLH) proteins. The Id gene family is involved in regulation of cell-cycle status and differentiation during embryogenesis and has been found in a rare type of neural stem cells, the B1 type, where it is necessary for self-renewal. Expression of Id proteins has been demonstrated in a variety of human tumors including gliomas and has been investigated as a potential proto-oncogene. Overexpression of Id1 in human tumor cells induces cell proliferation and invasion, and also protects cells against drug-induced apoptosis.	Kamalian et al., 2008; Maw et al., 2009; Nam & Benezra, 2009; Schindl et al., 2001; Schindl et al., 2003; Schoppmann et al., 2003; Tang et al., 2009
NG2	NG2 is a transmembrane proteoglycan that interacts with the ECM to mediate cell adhesion and proliferation. It is expressed on oligodendrocyte precursor cells in the adult CNS. It has been found in human acute myeloid leukemia and in gliomas, where it in the latter seems to increase tumor cell proliferation in vitro and promote angiogenesis in vivo.	Brekke et al., 2006; Chekenya et al., 1999; Chekenya et al., 2002a; Chekenya et al., 2002b; Chekenya et al., 2008; Chekenya & Immervoll, 2007; Chekenya & Pilkington, 2002; Joo et al., 2008; Petrovici et al., 2010; Stallcup & Huang, 2008
CD15	CD15 (leukocyte cluster of differentiation 15) also known as LeX or stage-specific embryonic antigen 1, SSEA-1, is an extracellular matrix-associated carbohydrate. CD15 is secreted by neural progenitor cells including stem cells into the stem cell niche, where it binds factors such as WNT-1 that are important for progenitor proliferation and self-renewal. It is highly expressed on pluripotent stem cells and has been found in CNS germinal zones. It has been found in various normal tissues but also in different cancers including gliomas.	Capela & Temple, 2002; Capela & Temple, 2006; Read et al., 2009; Ward et al., 2009

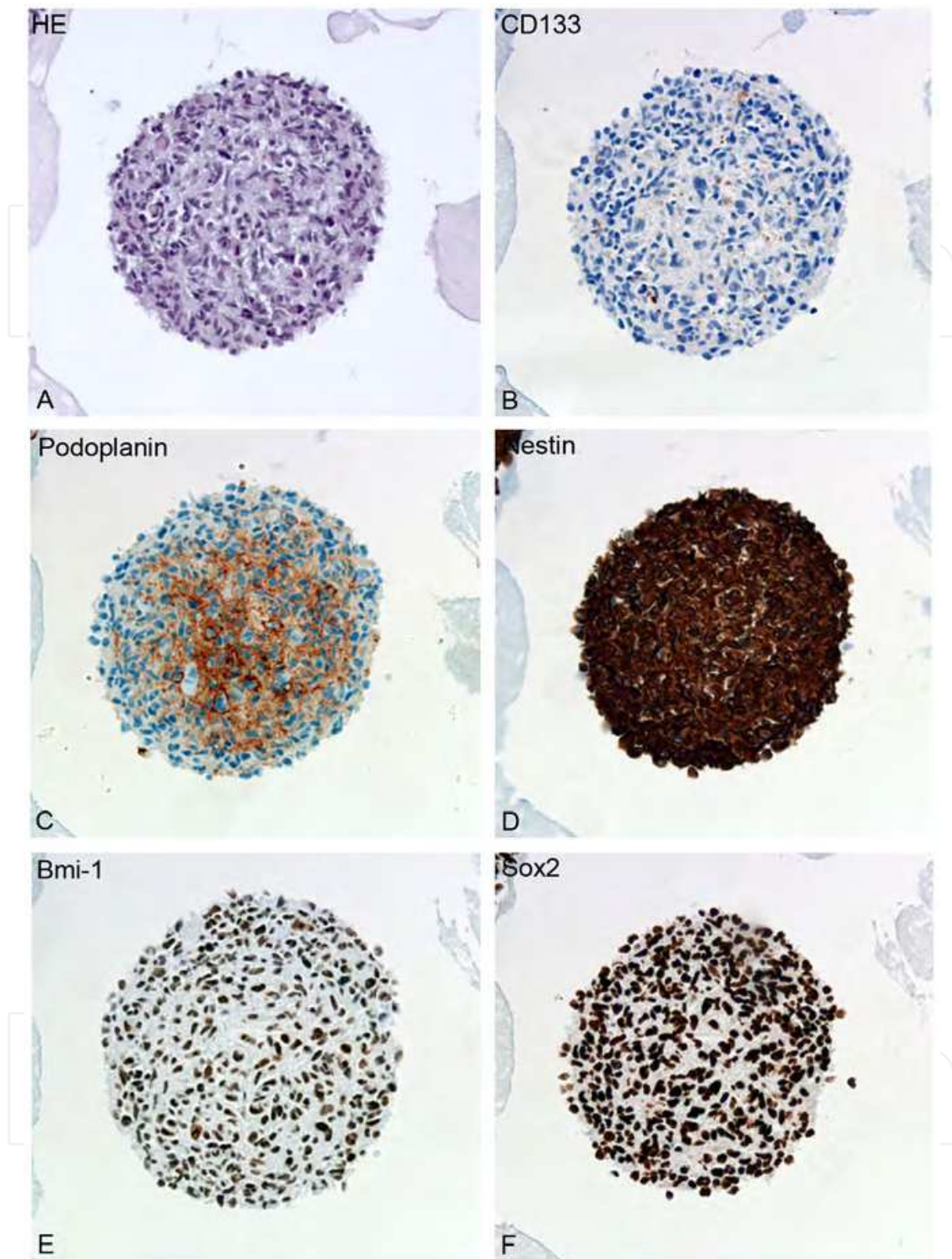Table 1. Some of the most used stem cell markers in the field of brain tumors.

Fig. 2. Spheroids derived from a glioblastoma short term culture were stained immunohistochemically with a panel of stem cell markers. After culturing, the spheroids were formalin fixed, paraffin embedded and sectioned in 3 μm thin sections followed by immunohistochemical staining. The section in (A) was stained with hematoxylin and eosin (HE) which is widely used in histology to identify cell nucleus and cytoplasm. Moreover, sections were immunohistochemically stained with the stem or tumor stem cell markers CD133 (B), Podoplanin (C), Nestin (D), Bmi-1 (E) and Sox2 (F).

CD133 was initially identified as a marker of hematopoietic stem cells in 1997 (Miraglia et al., 1997; Yin et al., 1997) and later as a marker of human neural stem cells (Uchida et al., 2000). In 2003 a CD133+ subpopulation of cells with stem cell properties were isolated from medulloblastomas and pilocytic astrocytomas by flow cytometry (Singh et al., 2003). The isolated CD133+ cells formed primary neurospheres in vitro, whereas the CD133- cells did not. As previously mentioned, the sphere forming capability is believed to be a stem cell hallmark. In 2004 the same group isolated CD133+ subpopulations from medulloblastomas and glioblastomas and showed that they also exhibited stem cell properties in vivo. The CD133+ population could initiate phenotypically similar tumors, when injected intracranially into NOD/SCID mice in numbers as few as 100 CD133+ cells. This was not the case for CD133- cells, where up to 100,000 cells could not initiate new tumor formation (Singh et al., 2004). Although these results had a great impact on the field of glioma research, it should be mentioned that today controversies exist in this area. An important paper contributing to this controversy was a paper showing that also CD133- cells were tumorigenic and could give rise to CD133+ cells (Wang et al., 2008).

In accordance with the general idea of neural stem cells residing in discrete stem cell niches in the adult subventricular zone (Riquelme et al., 2008; Zhu et al., 2005), we have found CD133+ cells in this particular zone (Hermansen et al., 2011). In line with this, immunohistochemical studies performed by different groups (Calabrese et al., 2007; Thon et al., 2010; Zeppernick et al., 2008) including our group (Christensen et al., 2008; Hermansen et al., 2011) have shown that CD133 is located in clusters or niches in brain tumors, some of which are perivascular. The size of the niches varies from large positive areas to small perivascular niches comprising only a few cells. Several studies, including studies from our group (Christensen et al., 2008; Hermansen et al., 2011; Immervoll et al., 2008) have, however, also reported a widespread CD133 expression pattern in areas of various normal tissues, which is not normally associated with stem cells. This suggests that CD133 is not specific for stem cells and should be used in combination with other stem or progenitor cell markers to isolate tumor stem cells.

4. Hypoxia and tumor stem cells

Several studies associate tumor hypoxia with poor patient outcome and resistance to therapies (Bar, 2011; Li et al., 2009; Mashiko et al., 2011). In line with this, one of the hallmarks of glioblastomas is the presence of necrosis, occurring as a consequence of poor oxygenation and nutrition because of rapid tumor growth and formation of vessel thrombosis (Hulleman & Helin, 2005; Louis et al., 2007; Preusser et al., 2006). The hypoxia-inducible factors (HIFs) are transcription factors upregulated at low oxygen levels. These factors mediate the cellular hypoxia response influencing angiogenesis, cell survival, chemotherapy and radiation resistance, invasion and metastasis (Bar, 2011).

Usually culturing of cells is performed at 21 % O_2, but with the knowledge that the physiological oxygen concentration in the healthy brain ranges between 2.5 % and 12.5 % O_2 and in glioblastomas is even lower (Bar, 2011), it is worth considering culturing cells at lower oxygen concentrations. Spheroids of large sizes become hypoxic even if cultured in normoxia because of a diffusion gradient. However, a study by Glicklis et al. (Glicklis et al., 2004) has described that hepatocyte spheroids with diameters up to 100 µm have a good oxygenation status. Other studies by Fehlauer et al. (Fehlauer et al., 2005; Fehlauer et al., 2006; Fehlauer et al., 2007) have reported that by using glioma spheroids with diameters of 200-250 µm, there are only few hypoxic cells and no central necrosis present.

Low oxygen levels in different tumor types are believed to increase the population of tumor stem cells and to promote a stem-like state (Bar et al., 2010; Heddleston et al., 2009; Saigusa et al., 2011; Soeda et al., 2009; Wang et al., 2011; Xing et al., 2011; Yeung et al., 2011). This is similar to results obtained for embryonic stem cells showing that low oxygen levels promote maintenance of pluripotent potential, and maintenance of the cells in an undifferentiated stem cell state (Ezashi et al., 2005; Heddleston et al., 2009). The existence of tumor stem cells has been suggested to be restricted to perivascular niches and hypoxic areas within the tumor (Heddleston et al., 2009) explaining the poor outcome and therapeutic resistance seen in these hypoxic tumors. In addition to obtaining a more in vivo like metabolic milieu when culturing cells in hypoxic conditions, hypoxia also seems to promote the existence and propagation of tumor stem cells (Heddleston et al., 2009; McCord et al., 2009; Seno et al., 2009; Soeda et al., 2009). Several studies thus reported an increase in spheroid diameter, cell proliferation and number of spheroids (Heddleston et al., 2009; McCord et al., 2009; Soeda et al., 2009) when culturing spheroids in hypoxic compared to normoxic conditions. In a study from our group (Kolenda et al., 2010), spheroids obtained from a glioblastoma short term culture and the commercial glioblastoma cell line U87MG were cultured in both normoxia and hypoxia. Interestingly, a significant increase in the expression of the proposed stem cell markers CD133, Podoplanin and Bmi-1 was found in both types of spheroids when cultured in hypoxia. Furthermore, a study by Heddelston et al. (Heddleston et al., 2009) proposed that a phenotypic shift from non-stem to stem-like cells was obtained when culturing tumor cells in hypoxia. On the more mechanistic level, the spheroid formation in hypoxia has been shown to be affected by the hypoxia inducible factors as shown in studies by Li et al. (Li et al., 2009) and Méndez et al. (Mendez et al., 2010). Knockdown of HIF altered spheroid formation in glioma spheroids, resulting in smaller and fewer spheroids. Overall these findings suggest that culturing of cells in hypoxia as spheroids provides important in vivo-like conditions that are optimal when studying the stem cell biology of brain tumors.

5. Primary spheroids and radiotherapy

In the last three decades radiotherapy has been the standard treatment or part of the standard treatment for newly diagnosed glioblastoma patients (Stupp et al., 2009) providing a significant survival benefit (Laperriere et al., 2002). However, due to resistance to the current treatment of a subset of cells, it remains palliative. Primary spheroids obtained from glioma tissue have for years been reported to be a useful model for investigating in vitro radiobiology due to the preserved cellular organization. Features existing in these spheroids such as cell-cell contact, variation in the cell cycle distribution, diffusion effects, altered metabolism and hypoxia may influence the outcome of treatment, contributing to a better resemblance of the in vivo situation than obtained with a monolayer model (Olive & Durand, 1994; Sutherland & Durand, 1972). One feature of particular importance in these spheroids is possibly the low oxygen status being partly responsible for the increased radioresistance of the spheroid tumor cells (Blazek et al., 2007; Hsieh et al., 2010; Sutherland, 1998). Ionising radiation causes the formation of reactive oxygen species (ROS) (Brahme & Lind, 2010; 2008) and oxygen has therefore long been known to be a potent radiosensitizer (Vlashi et al., 2009). ROS causes damage to cellular components including DNA damage (Nishikawa, 2008) and are critical for irradiation-induced killing of tumor cells (Diehn et al., 2009). However, there have also been reports of no evident correlation between hypoxia and radioresistance (Buffa et al., 2001; Gorlach & Acker, 1994; Sminia et al., 2003) A study by

Sminia et al. (Sminia et al., 2003) found that both hypoxic and well-oxygenated organotypic multicellular spheroids derived from glioblastoma specimens showed high resistance to irradiation.

A study by Kaaijk et al. (Kaaijk et al., 1997) described the observation of only minor histological changes including a few shrunken nuclei, but no major histological damage in normoxic organotypic multicellular glioblastoma spheroids after a single dose of 50 Gy. This is in line with a study by Bauman et al. (Bauman et al., 1999), where C6 astrocytoma spheroids were implanted into a collagen type I gel. Following irradiation with 12 and 25 Gy, neither the hypoxic core nor the rim of the spheroids experienced a significant increase in the fraction of apoptotic cells. Similar to this, U87MG monolayer cultures irradiated with 8 and 20 Gy showed no considerable apoptosis five days after treatment and remained viable ten weeks after a 40 Gy dose was administered. However, in fact Kaaijk et al. reported that proliferation in three investigated organotypic multicellular spheroids was decreased 7-20 fold relative to untreated controls one week after hypofractionated radiation with a total of 40 Gy. Moreover, Fehlauer et al. (Fehlauer et al., 2005; Fehlauer et al., 2006) described in two studies a decrease in the percentage of MIB-1 positive proliferative cells in organotypic multicellular spheroids following irradiation with 20 Gy.

It has also been suggested that tumor stem cells might show increased radioresistance compared to more differentiated cells (Bao et al., 2006; Phillips et al., 2006; Rich, 2007). Bao et al. (Bao et al., 2006) thus showed that CD133+ cells survived ionizing radiation better than CD133- cells and that the fraction of CD133+ cells was enriched in gliomas after radio-therapy, suggesting that the CD133+ cellular population of gliomas is contributing to glioma radioresistance and could be the source of tumor repopulation after radiation. Liu et al. (Liu et al., 2006) investigated mRNA levels of various markers including BCRP1 (breast cancer resistance protein), MGMT (O-6-methylguanine-DNA methyltransferase), anti-apoptosis proteins and inhibitors of apoptosis protein families in CD133+ cells isolated by FACS. These markers are involved in treatment resistance and elevated mRNA levels were shown in CD133+ cells compared to CD133- cells. A significant degree of resistance towards chemotherapeutics such as temozolomide, carboplatin, paclitaxel and etoposide were demonstrated in the CD133+ cells (Liu et al., 2006). In line with these results Liu et al. showed enrichment of CD133+ cells in five recurrent gliomas when compared to the respective newly diagnosed tumors. Furthermore, results obtained in our laboratory have shown a much more pronounced reduction in the secondary spheroid formation capacity of irradiated spheroids derived from recently established glioma spheroids with stem cell characteristics compared to U87MG derived spheroids without these characteristics (Jakobsen et al. 2011).

6. Spheroids and chemotherapy

Besides irradiation and surgery, the treatment of glioblastomas consists of chemotherapy. Although the introduction of temozolomide as standard chemotherapeutic in 2005 (Stupp et al., 2005) has increased the overall patient survival, new and more efficient chemotherapeutics or targeted therapies are urgently needed. Here spheroids also have an important role to play.

Investigations of the specific effects of chemotherapeutics and other drugs on glioma spheroids are often done by investigating the size and number of spheroids as well as the

viability and the proliferation of cells in the spheroids, including the ability of the cells to form secondary spheroid.

Frequently used assays for measuring the viability of the cells after treatment is tetrazolium-based cell proliferation assays. Several variations of this assay exists (XTT, MTT, MTS or WST-1) (Berridge et al., 2005), but all utilize the conversion of tetrazolium salts by active mitochondria into dark red formazan that can be monitored by absorbance measurements (Berridge et al., 2005). Usually these assays are used on adherent monolayer cultures, which consist of uniform cell populations. However, these assays have also been used on spheroids consisting of more heterogenous cell populations. In one study (Johannessen et al., 2009) the doxorubicin sensitivity was determined in high and low passage spheroids by a MTS-assay. This was done by placing one spheroid per well in a 96 well plate, measuring viability relative to size after incubation with doxorubicin for 96 hours. After the viability measurements, the spheroids were allowed to adhere to the bottom of the plastic plates resulting in cell migration from the spheroids. Immunostaining of the migratory cells were performed using the neural stem cell markers Nestin, Vimentin and Musashi-1.

Another widely used assay is the lactate dehydrogenase assay (LDH-assay), measuring cell death. The LDH-assay indirectly measures plasma membrane damage, which is related to cell death. Due to membrane damage, LDH leaks to the culture medium, where it participates in the conversion of tetrazolium salts to formazan. The amount of formazan produced is directly proportional to the amount of LDH in the culture medium, which in turn is directly proportional to the number of dead or damaged cells (Korzeniewski & Callewaert, 1983).

The size of spheroids after drug treatment has also been used as a measure of cell viability (Fehlauer et al., 2007; Johannessen et al., 2009; Khaitan et al., 2009; Yamaguchi et al., 2010). Khaitan et al. (Khaitan et al., 2009) investigated the effect of the glycolytic inhibitor 2-deoxy-D-glucose on spheroids derived from a human glioma cell line by measuring the size of the spheroids after drug exposure. In a stem cell context, number and size of primary and secondary spheroids have also been widely used as measures of the self-renewal potential, which is one of the hallmarks of stem cells. Especially the traits that are attributable to tumor stem cells are of interest, as the tumor stem cell hypothesis states that the tumor stem cells need to be targeted specifically in order to improve cancer treatment. In the so called spheroid formation assays or clonogenic assays, the ability of the cells to form spheroids is investigated. In many studies (Sunayama et al., 2010; Wakimoto et al., 2009; Wang et al., 2010; Zhu et al., 2010) this is primarily done after treatment of the cells, thereby investigating the effect of a given drug on the ability of the cells to self-renew. Different experimental setups have been employed using different cell densities, probably resulting in two different assays - one assay with high cell densities for evaluation of proliferation and another assay with small so-called clonal cell densities for evaluation of self-renewal or clonogenic capabilities of the cells. High cell densities often result in cell and spheroid fusion due to a high motility of the spheroids (Singec et al., 2006) and it is therefore not possible to investigate the self-renewal mechanism in this assay. A plating density of 20 cells/μl has been considered as clonal conditions in terms of neurosphere formation (Singec et al., 2006). In glioma studies cell densities ranging between 0.15 cells/μl to 300 cells/μl have been used (Kolenda et al., 2010; Sunayama et al., 2010; Wakimoto et al., 2009; Wang et al., 2010; Zhu et al., 2010) when studying spheroid formation.

7. An in vivo-like in vitro model of glioma invasion

Gliomas are known to be highly invasive and new knowledge concerning tumor cell invasion also incorporating the tumor stem cell aspect is urgently needed. In our laboratory we have worked to improve in vitro models when investigating the invasive features of glioblastoma cells. This led to establishment of an in vivo-like model of invasion, where spheroids are implanted into organotypic brain slice cultures.

The organotypic brain slice cultures became very popular research tools especially with the development of the roller-tube technique by Gähwiler in 1981 (Gahwiler, 1981) and the inter-face culturing method developed by Stoppini in 1991 (Stoppini et al., 1991). These cultures preserve many of the basic structural and connective tissue structures present in the tissue, when it is localized in the brain (Gahwiler, 1988). By implanting the organotypic spheroids into the organotypic brain slice cultures, it is possible to establish an organotypic spheroid-based slice-culture invasion assay, suitable for following tumor cell invasion into the brain tissue in vitro.

The investigation of glioma invasion has been performed since the 1980's but the models used have improved during the years. Different assays have been used to address the invasive capacities of the tumor cells. A frequently used migration assay allows spheroids to adhere to the bottom of coated plastic plates, and after a period of time the distance of migrating cells from the spheroid can be measured (Gliemroth et al., 2003; Narla et al., 1998; Terzis et al., 1997; Terzis et al., 1998). Another extensively used invasion assay is the Boyden or Boyden-like chamber-based assays. The Boyden chamber was first introduced by Boyden in order to investigate the chemotactic effect of mixtures of antibody and antigen on leukocytes (Boyden, 1962). The principle in the Boyden chambers is cell migration into a microporous membrane, often made of matrigel (Deryugina et al., 1997; Paulus & Tonn, 1994; Schichor et al., 2005). The membrane is placed in between two medium-filled compartments; the upper compartment containing cells whereas the lower compartment may contain a chemotactic agent. After an incubation period, the cells migrating through the microporous membrane can be stained and counted (Chen, 2005). In a variation of the Boyden Chamber assay, slices of porcine white and gray matter were placed on top of a filter between the two compartments facilitating the cells to migrate through the porcine brain slice, making this assay a combination of the Boyden Chamber and the organotypic co-culture system (Schichor et al., 2005).

In the first real invasion studies, tissue aggregates from rat brain or chick heart were (Lund-Johansen et al., 1990) placed next to the tumor tissue, but first with the development of the organotypic brain slice culture, it was possible to preserve the brain architecture and organization in an optimal way, thereby creating the conditions necessary for a more in vivo-like model of glioma invasion. Several groups have been using this model to investigate the invasion of glioma cell into organotypic brain slice cultures (Aaberg-Jessen et al. 2011; Caspani et al., 2006; De et al., 2002; Eyupoglu et al., 2005; Guillamo et al., 2009; Jensen et al. 2010; Matsumura et al., 2000; Ohnishi et al., 1998; Palfi et al., 2004; Stoppini et al., 1991). In one study using this model, invasion was shown to be associated with the histological type and grade of the tumor (Palfi et al., 2004) and in another study invasion and tumor-induced neurotoxicity was shown to be associated (Eyupoglu et al., 2005). Most interestingly, quantitative analysis of invasion has also been performed (De et al., 2002) using confocal laser scanning microscopy and a three-dimensional visualization after having followed invasion over several weeks (Matsumura et al., 2000).

Besides investigating tumor invasion into the brain tissue, the model offers several other applications. Guillamo et al. (Guillamo et al., 2009) investigated the invasion, proliferation and angiogenesis of six human malignant glioma spheroids implanted into organotypic brain slice cultures, when these co-cultures were treated with gefinitib. Some of the tumors implanted had EGFR amplifications resulting in more pronounced invasion than tumors without EGFR amplification. Upon treatment with gefinitib only tumor cell invasion from tumors with EGFR amplification was inhibited, whereas vascular density was decreased in all tumors. In another study, Caspani et al. (Caspani et al., 2006) used the co-culture model to investigate the re-organization of the cytoskeleton in migrating glioblastoma cells. Cells transfected with green fluorescent protein were introduced into collagen gels, brain slice cultures and in vivo into mice brains and the re-organization and motility of the glioblastoma cells in the different models were monitored by confocal microscopy.

Organotypic brain slice cultures from rodents have been used in a variety of different studies using tissue obtained from different areas in the brain. The organotypic brain slice cultures used in the invasion studies varied from explants from spinal cord (Caspani et al., 2006), brain slices cut in the sagittal plane (Caspani et al., 2006) and the coronal plane (De et al., 2002; Guillamo et al., 2009; Matsumura et al., 2000; Ohnishi et al., 1998; Palfi et al., 2004) as well as entorhinohippocampal slice cultures (Eyupoglu et al., 2005). Since glioblastomas are often located in the subcortical white matter of the cerebral hemisphere and tumor infiltration often extends into the adjacent cortex and through corpus callosum (Louis et al., 2007) we have used organotypic corticostriatal brain slice cultures with cortex, striatum and corpus callosum for our studies of the invasive features of the glioblastoma cells. Such corticostriatal slice cultures should be cultured by the interface method placing the brain slices at the interface between air and culturing medium on a porous, transparent and low-protein binding membrane. This allows the cultures to be oxygenated on one site, while receiving nutrients from the other site. In the following text our approach is described in details.

The corticostriatal slice cultures are prepared from newborn Wistar rat pups by a method slightly modified from Kristensen et al. (Kristensen et al., 1999). The brain is aseptically removed from the pup and placed in a petri dish under a stereomicroscope, where the meninges are carefully removed. Hereafter the brain is sectioned coronally in 400 µm slices on a McIIwain tissue Chopper and the slices are transferred to a petri dish containing Hanks' Balanced Salt Solution supplemented with 0.9% glucose. The brain slices are separated from each other and the sections containing cortex and striatum are divided into the two hemispheric parts resulting in the final brain slices. These slices are randomly moved to the insert membranes with four cultures on each membrane. Finally, the membrane inserts are placed in a 6-well plate in 1 ml preheated medium, and incubated in 36°C humidified air containing 5% CO_2 and 95% atmospheric air.

Cell line spheroids or organotypic primary spheroids are implanted into organotypic brain slice cultures in the corpus callosum area between cortex and striatum, whereby a co-culture is established. In order to identify and follow tumor cells when they invade the slice, the spheroids can be labeled with the fluorescent dye, DiI (1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) for 24 h before implantation, enabling confocal microscopy. The labeled spheroids are prepared by adding a DiI solution to the medium with spheroids, achieving a concentration of 25 µg/ml. Before implantation the spheroids are washed in medium to avoid bringing excess dye onto the brain slice cultures. Spheroids around 200-400 µm in diameter are captured using a denudation pipette and

thereafter carefully placed next to corpus callosum between cortex and striatum in the brain slice cultures. The culturing plates are placed in the incubator in 36°C humidified air containing 5% CO_2 and 95% atmospheric air, whereafter the medium is changed twice a week. By monitoring the co-culture with confocal time-lapse microscopy, tumor cell invasion into the surrounding rat brain tissue can be visualized. Using a confocal microscope, a z-stack can be made consisting of thin sections at different levels of the culture. This makes it possible to follow invasion from all parts of the spheroid in different layers of the brain slice culture, whereby a three-dimentional movie and image can be constructed or alternatively an accumulated two-dimensional image based on overlay of all the images from one z-stack.

1. Decapitate a newborn rat pup
2. Aseptically remove the brain
3. Remove the meninges
4. Section the brain coronally in 400 μm slices on a McIIwain tissue Chopper
5. Separate the brain slices and choose the slices containing cortex and striatum
6. Divide these slices into the two identical halves for obtaining the final brain slice cultures
7. Move slices randomly to a transparent insert membrane
8. Place the insert membranes in a 6-well culturing plate with 1 ml preheated medium
9. Incubate the cultures in 36°C humidified air containing 5% CO_2 and 95% atmospheric air
10. Change the medium twice a week
11. Label organotypic spheroids or spheroids derived from short term cultures with 25μg/ml DiI for 24 h before implantation
12. Wash the spheroids 3 times with medium before implantation
13. Use spheroids in the size range from 200-400 μm
14. Place the spheroids in the area between cortex and striatum next to corpus callosum by a denudation pipette
15. Incubate the co-cultures in 36°C humidified air containing 5% CO_2 and 95% atmospheric air
16. Monitor the DiI-labeled spheroids using confocal microscopy

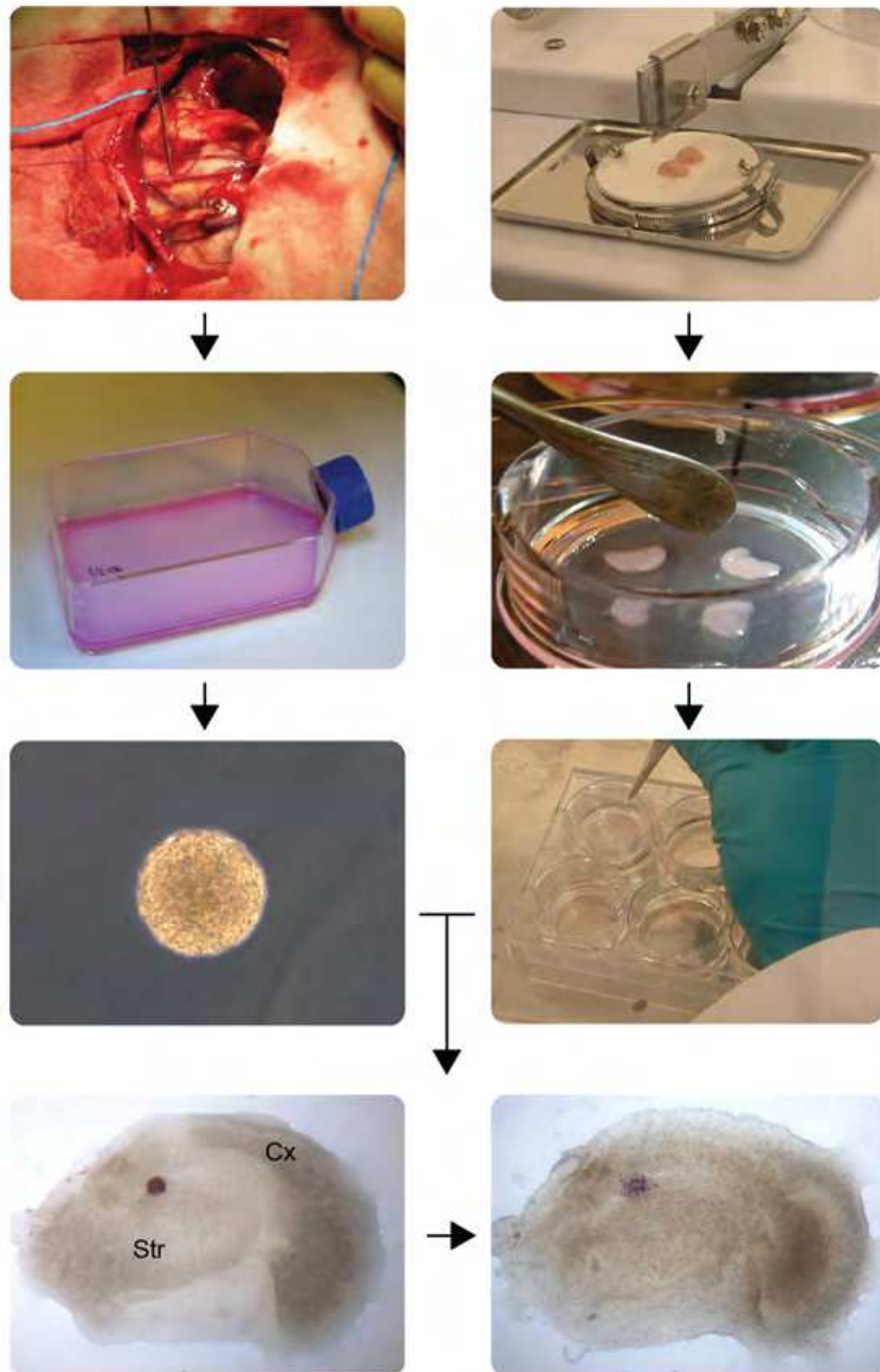Box 2. Preparation of organotypic corticostriatal brain slice cultures and implantation of spheroids.

Fig. 3. Schematic overview of the implantation of glioma spheroids into organotypic rat corticostriatal brain slice cultures. Glioma tissue is obtained from patients and collected in the operation theatre. Thereafter, it is processed and cultured in the laboratory until spheroids are formed. Alternatively, spheroids from established short term cultures or cell lines can be used. Simultaneously, brain slice cultures from newborn rats are prepared and cultured by the interface method. The spheroids are labeled with the fluorescent dye DiI and implanted into the brain slice cultures in the corpus callosum area between cortex and striatum, here illustrated by a phase contrast image of a spheroid immediately after implantation and after 14 days of culturing. Note the less marked edge of the spheroid as the cells migrate into the surrounding brain tissue (Cx- cortex and Str-striatum).
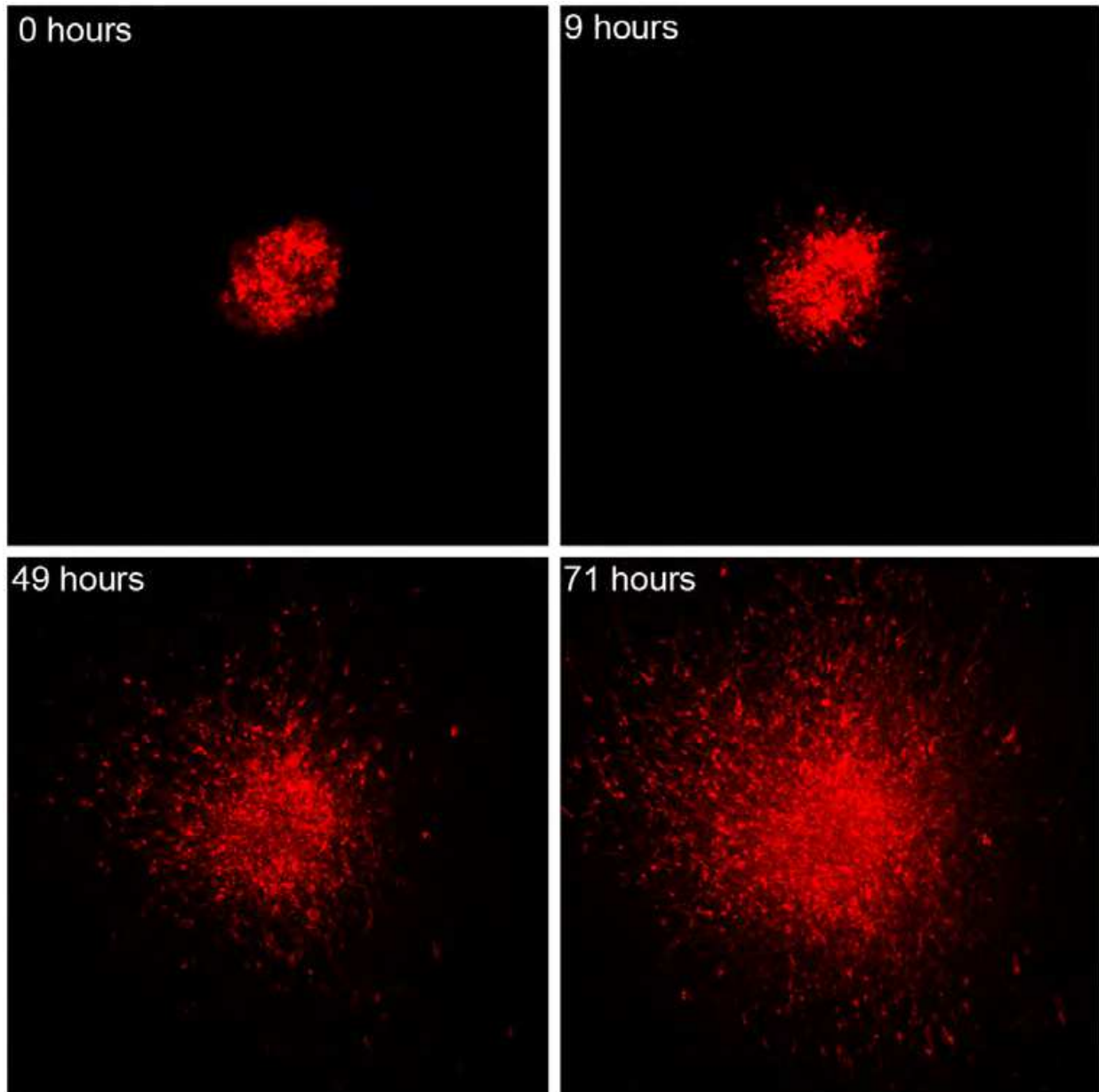
Fig. 4. Confocal images of an invasive DiI-labeled glioma spheroid implanted into an organotypic rat brain slice culture. The spheroid is followed by confocal timelapse microscopy for a period of 72 hours. The images are accumulated images based on overlay of all the images from one z-stack. A z-stack consists of several images obtained at different levels of the co-culture.

8. Conclusion

We conclude that the three-dimensional spheroid models offers advantages in glioma research taking tumor biology and microenvironment into account. Especially, when using the organotypic models, where the structure and organization of the tissue is preserved, features close to the in vivo situation are supposed to be obtained. In tumor stem cell research, the spheroids are a necessary tool as this culture method seems to promote the existence of these cells. This is especially the case when culturing the spheroids in a hypoxic

environment. As discussed in the chapter, spheroids have been used in a wide range of experiments investigating radiation responses, effects of chemotherapy and effects of different types of experimental drugs as well as in migration and invasion studies. The experimental setups may be somewhat more difficult than by using monolayer cultures, but since the spheroid models are supposed to be closer to the in vivo situation, the results and answers obtained are also supposed to be closer to what it true for the corresponding tumors in the human brain. However, efforts should be made to develop these three-dimensional models to become even more in vivo-like, in order to meet new challenges in glioma research and drug development. The use of spheroids in especially tumor stem cell research has been fast increasing in recent years making spheroids an important tool also in future glioma research.

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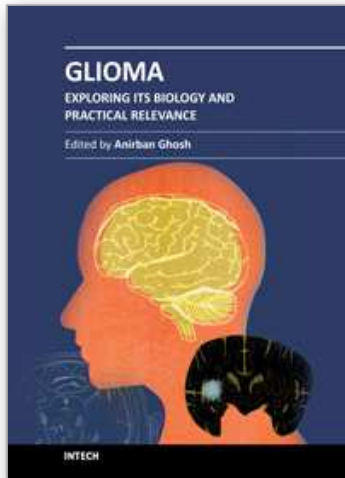
Glioma - Exploring Its Biology and Practical Relevance

Edited by Dr. Anirban Ghosh

The tittle 'Glioma - Exploring Its Biology and Practical Relevance' is indicative of its content. This volume contains 21 chapters basically intended to explore glioma biology and discussing the experimental model systems for the purpose. It is hoped that the present volume will provide supportive and relevant awareness and understanding on the fundamental advances of the subject to the professionals from any sphere interested about glioma.

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