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New therapeutic and diagnostic strategies in glioblastoma:

Cancer Stem Cells targeted by monoclonal antibody

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A special thank to my family that encourage me when I am in the dark moment, that makes easy all the difficulty and give me the power to proceed.

To my parents

CONTENTS

SUMMARY

One of the most challenging objective in oncology is the identification of new specific tumor target to design effective therapies.

At present it is widely accepted that the tumors are originated from a undifferentiated cell population able to trigger the cancer establishment and responsible for its maintenance. These undifferentiated cells are called Cancer Stem Cells (CSC) or tumor-initiating cells. A direct consequence of the CSC hierarchical model applied to clinic is that is currently taking place the idea that the traditional cancer treatment concern only the bulk tumor and the differentiated population without affecting the stem cell compartment. In that scenario the resistant cancer stem cells can proliferate and diffuse resulting in a temporary success of the treatment followed by a relapse. For this reason an effective therapy should be addressed to the eradication of CSCs that represent a gold target from the clinical treatment. CSCs are relatively slow proliferating in vivo, where they divide asymmetrically and are amplified few time with a massive duplications cycles of division made by progenitors. Therefore CSCs can be resistant to several available therapies which primarily affect actively duplicating cells. Despite to their relative quiescence, several mechanisms could account for this resistance such as the increased expression of drug pumps (Zhou, Schuetz et al. 2001), the expression of anti-apoptotic proteins, the higher efficiency in repairing DNA damage (Bao, Wu et al. 2006).

The aim of this work was to specifically target CSCs of one of the most important causes of death for cancer, glioblastoma multiforme (GBM) by generating monoclonal antibodies (mAbs). Glioblastoma multiforme is the most common and most aggressive malignant primary brain tumor in humans. Even after treatments based on multiple approaches, as radiotherapy, surgical resection and chemotherapy, the prognosis of GBM patients remains unfavorable. The generation of mAbs against new surface markers against glioblastoma stem cells could have an impact as for the diagnostic as for therapeutic application. New mAbs could help to set up new diagnostic tests or to follow the effect of therapies. The mAbs generated could be employed as new therapeutic tools with selective toxicity against tumor cells, add efficacy to present therapies and represent an additional line of treatment for patients in advanced stages with multiresistant diseases. Moreover the discovery of a common stemness marker as antigen on glioblastoma stem cells may allow to select for cancer stem cells and this

help to isolate and characterize them and possibly may be useful for defining novel diagnostic techniques and targeted therapies.

The glioblastoma stem cells maintain the characteristics of tumor initiating cells after prolonged culture in vitro. We used these cells as immunogens in immunocompetent BALB/c mice to develop a hybridoma library. After the initial screening of the library was identified the 1.4A12 antibody that identified integrinα7 (ITGA7) as antigen mostly expressed on brain tumor stem cells. The biological function of the integrinα7 was evaluated the vitro and in vivo. ITGA7 silencing impairs the growth rate proliferation, clonogenicity, invasion in vitro and the tumor growth reduction in vivo. We analyzed the effect of 1.4A12 antibody and its role in counteracting the ITGA7 pathway. The 1.4 anti-integrin alpha7 antibody interferes with the integrin alpha 7 signaling and suppressed tumor invasion in vitro and tumour growth in vivo. All our data indicated that integrin alpha7 is involved in GBM pathogenesis and that 1.4A12 antibody could be of great help in defining new therapeutic options to increase glioblastoma eradication interfering with the tumor growth and with the invasion of cancer cells.

INTRODUCTION

1 MONOCLONAL ANTIBODIES

1.1 Monoclonal antibodies features

Over a hundred years have passed from the discovery of the "magic bullet" serum therapy by Behring that led to the awarding of the Nobel Prize in Medicine and Physiology in 1901. Behring and his group demonstrated that the transfer of serum from a guinea pig immunized with diphtheria toxin to another guinea pig offered protection from the toxin. Over the years the serum, from immunized non-human sources (horses or rabbits), was applied to care tetanus toxin bacterial diseases including diphtheria, meningitis, and pneumonia, demonstrating the action of this system. Many foreign proteins contained in the serum gave a phenomenon that has been called "serum sickness". Behring recognized these toxic side effects of serum therapy and introduced improved methods for the purification of serum. Köhler and Milstein developed methods for the isolation of mAbs (Monoclonal Antibodies) from hybridoma cells in 1975 (Kohler and Milstein 1975). They showed that the cell fusion technique could be used to produce hybrids between myeloma cells and antibody producing cells. The resulting hybrid lines were permanently adapted to grow in tissue culture and were capable of inducing antibody production in mice (Jerne and Nordin 1963). This discovery led to the awarding of the Nobel Prize in Physiology or Medicine in1984 to Köhler and Milstein. Köhler and Milstein's method opened the use of hybridoma technology to academics and pharmaceutical fields for generation of antibodies for therapy. The first therapeutic mAb generated was OKT3 by Ortho Biotech in 1984 against CD63 antigen expressed on T cells for the treatment of transplant rejection. Initially, mAbs were from mouse, leading to a relative immunogenic capacity in humans. This issue was progressively solved with the increasing substitution of murine sequences with human ones, which led to the development of chimeric or fully humanized mAbs. The chimeric antibodies have one-third murine sequences (2 VH and 2 VL subunits) fused with two-thirds human sequences, including the human Fc part(Morrison, Johnson et al. 1984) (Boulianne, Hozumi et al. 1984). The humanized Abs have the V chains from a murine, or other mammalian antibody, "more human". Antibodies humanized reduce the human immune response against this murine antibody for the production of human antimouse antibody (HAMA).
The humanized antibodies have the complementarity-determining regions (CDRs) of murine antibodies fused into the closely related human structure, followed by aminoacid changes

required to stabilize the engineered constructs (Jones, Dear et al. 1986). The first humanized mAb used as a therapeutic agent was an anti-CD25 (IL-2 alpha subunit) mAb that was humanized and developed to suppress rejection after transplantation from Quen group (Queen, Schneider et al. 1989). In contrast to these in vitro manipulations for humanizing mAb, two research groups independently developed functional human mAbs from "humanized" transgenic mice. The mice used were unable to produce their own murine antibodies and replacing that function with human antibody genes (Green, Hardy et al. 1994; Lonberg, Taylor et al. 1994). These transgenic humanized mice were immunized with an antigen and fully human antibodies were generated in these mice. The first fully human mAb developed from these humanized systems was Panitumumab, a human IgG2 antibody discovered using Abgenix XenoMouse technology, against EGFR.
Other group proposed another approach to develop antibodies. Smith's group reported that peptides were able to be displayed as fusions of P3 protein on the tail fibers of filamentous phage M13 (Smith 1985) (Barbas, Kang et al. 1991). This method was applicable for the display of proteins including mAbs (Barbas, Kang et al. 1991). This M13 P3-based phage display technology has become an optimal methodology for selection of antibody fragments (Gram, Marconi et al. 1992). McCafferty et al. reported that was possible build a library of antibody genes, displayed on the P3 protein of M13 phage, using PCR techniques to recover the human gene from either B cells or hybridomas (McCafferty, Griffiths et al. 1990). This method was followed by the construction of huge human libraries from either synthetic repertoires or from multiple naïve human donors (Barbas, Bain et al. 1992) (Marks, Hoogenboom et al. 1991).

The antibody-based therapy have been developed to target the tumor cells but also to enhance the antitumor immune responses. Many mechanisms have been proposed to explain the antitumor activity of monoclonal antibodies as antibody-dependent cellular cytotoxicity (ADCC) (Fig 1a) and complement-dependent cytotoxicity (CDC). ADCC is initiated by the recognition of Fc part of IgG-coated tumors by Fc receptors (FcγRs) of natural killer (NK) cells, macrophages and neutrophils. These interactions lead to ADCC and to tumor cell apoptosis triggered by cytotoxic granules (containing perforin and granzyme B), released by effector cells. Most clinically approved monoclonal antibodies that mediate ADCC also activate the complement system. IgG can bind to tumor cells and recruit complement factors resulting in tumor cell lysis. Another antitumor mechanism mediated by IgG on tumor cells and FcγR receptor on macrophages interactions is the phagocitosis that induces the

lysosomal degradation of the tumor cell (Fig 1b). Dendritic cells (DCs) are also capable of presenting peptides from lysosomal degradation of tumor cells on MHC class I molecules to determinate antigen-specific CD8+ T citotoxic cell responses and on MHC class II leading to the activation of CD4+ T cells, which can activate B cells for the production of tumor-specific host antibodies (Fig 1c) (Weiner, Surana et al. 2010).

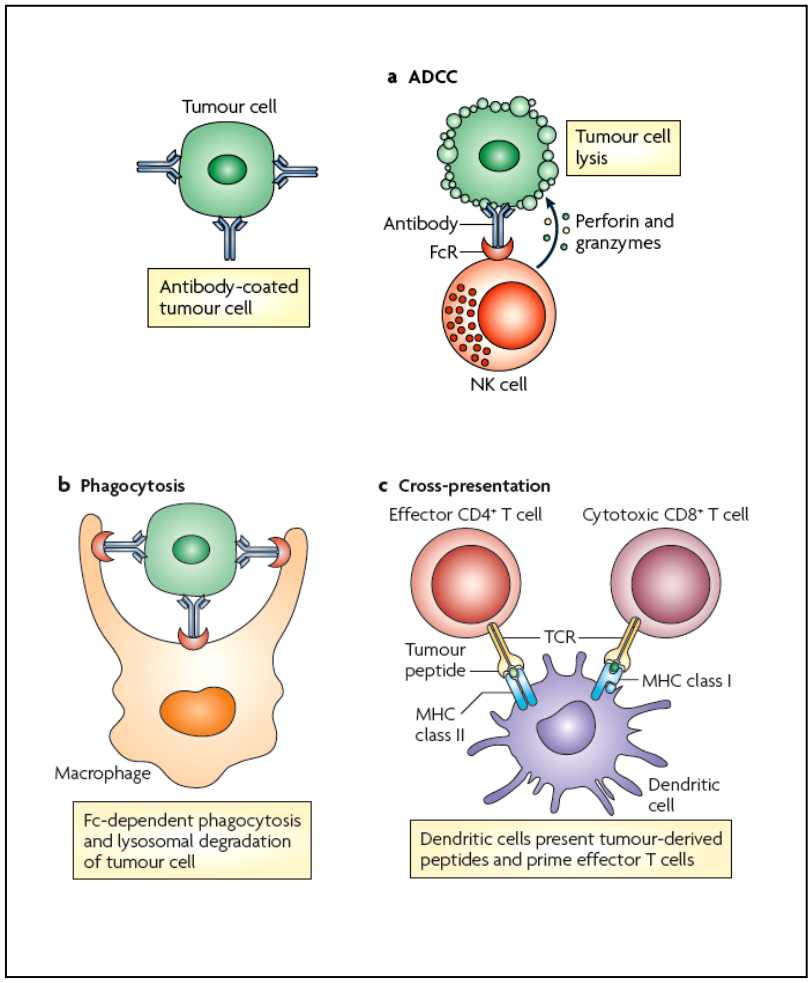


Fig 1: Antitumor mechanisms mediated by IgG–FcγR interactions **a**) Antibody-dependent cell cytotoxicity (ADCC) is promoted by the recognition of IgG-coated tumors by Fc receptors for IgG (FcγRs) leading to tumor cell apoptosis. **b**) Fc receptor expressed by phagocytes can bind IgG-coated tumor cells causing Fc-dependent phagocytosis and lysosomal degradation. **c**) Dendritic cells present peptides from lysosomal degradation of tumor cells on MHC class I molecules and MHC class II activating T cells.
(Weiner et al, 2010)

1.2 Oncologic application of Monoclonal antibodies

CD20

Rituximab was the first mAb to be approved by the United State Food and Drug Administration (FDA) for oncological treatment. Rituximab is a mouse-human chimeric mAb against CD20. It derived from mouse anti-CD20 mAb by engineering of human IgG1 and kappa constant regions and the original murine variable regions (Reff, Carner et al. 1994). CD20 is expressed in more than 90% of non-Hodgkin lymphomas and 10% of chronic lymphocytic leukemias. Anti-CD20 mAb is efficient in B cell malignancies treatment because CD20 is expressed at high levels on B cells, is relatively resistant to internalization, thereby allowing the mAb to persist on the cell surface. The binding between CD20 and rituximab induces apoptosis and cell lysis via complement-dependent cytotoxicity (CDC) and ADCC. Clinical studies of rituximab in relapsed or refractory CD20-positive non-Hodgkin B cell lymphomas showed that combination chemo/immunotherapy is superior to treatment alone (Reff, Carner et al. 1994).

Human epidermal growth factor receptor 2 (HER2)

The most known antibodies in solid tumors targeted therapy are the antibodies directed against the EGFR family. Anti-HER2 mAb trastuzumab (Genentech) was derived from murine mAb by humanization of human IgG1 and kappa constant regions fusing the murine complementarity determining regions (Carter, Presta et al. 1992). HER2 is a transmembrane receptor and is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. HER2 normally regulates cell growth and cell cycling via cyclin D and c-myc (Lane, Beuvink et al. 2000). HER2 gene amplification and/or overexpression occurs in around 20–30% of primary breast carcinomas and is associated with reduced survival (Press, Bernstein et al. 1997). Trastuzumab binds to an epitope in the juxtamembrane region of HER2 on breast carcinoma cells, acting as an “anti-cancer reagent” through a direct inhibition of cell growth and receptor internalization and degradation decreasing cell signaling progression. Trastuzumab inhibits cleavage of an extracellular domain induced by metalloproteinase, induces apoptosis and impairs tumor angiogenesis (Baselga, Albanell et al. 2001; Mohsin, Weiss et al. 2005) (Izumi, Xu et al. 2002). In metastatic breast carcinoma patients, trastuzumab combined with standard chemotherapy produces a better response rate, decreasing the time to disease progression, and overall survival compared with

chemotherapy or trastuzumab alone (Esteva, Valero et al. 2002). Better response rates has been observed in patient with overexpression of HER2 as compared to patients with normal expression (Seidman, Fornier et al. 2001). Trastuzumab was approved in 1998 for treatment of breast cancer with HER2 overexpression. The indication of FDA approval recently was as adjuvant treatment of patients with HER2-overexpressing tumors with lymph node metastasis as part of a regimen of doxorubicin, cyclophosphamide and paclitaxel. Trastuzumab is approved for use as a single reagent in patients with metastatic breast carcinoma whose tumors overexpress HER2 and who have received one or more chemotherapy regimens.

CD33

CD33 is a type I transmembrane sialoglycoprotein that is a member of immunoglobulin-like lectins superfamily (Crocker, Paulson et al. 2007). CD33 is expressed on the cell surface of myeloid and sialic acid -binding early multilineage hematopoietic progenitor cells, monocytes, and blasts of acute myeloid leukemia (AML) (90%). CD33 is not expressed on normal pluripotent stem cells or non-hematopoietic cells (Brendel and Neubauer 2000). Gemtuzumab ozogamicin (Mylotarg) is a humanized mAb against CD33 conjugated with cytotoxic anti-tumor antibiotic calicheamicin. Gemtuzumab is derived from murine IgG1 mAb generated with human IgG4 and kappa constant regions, while retaining complementarity determining regions of the murine mAb (Hamann, Hinman et al. 2002). Gemtuzumab ozogamicin binds to the cell surface of CD33-positive leukemic cells with an approximate affinity of 0.08 nM. This mAb acts as a vehicle delivering a conjugated toxic agent to CD33 positive leukemic cells. Binding of the mAb to CD33 results in endocytosis, cleavage of the link between mAb and calicheamicin, and release of calicheamicin that is reduced by glutathione to form a reactive intermediate that binds to DNA, generating a double-strand breaks and inducing apoptosis (Dedon, Salzberg et al. 1993). In a phase II study, 277 patients with CD33-positive AML received monotherapy with gemtuzumab ozogamicin. As a result, 26% achieved remission, 13% of the patients had complete remission (Larson, Sievers et al. 2005). The current FDA approved indications include treatment of patients with CD33-positive AML in first relapse who are more than 60 years old and who are not considered candidates for chemotherapy.

Vascular endothelial growth factor (VEGF)

VEGF is an angiogenic growth factor that is activated by the binding with the receptor tyrosine kinase VEGF receptor 2 (also call Flk-1or KDR). These receptors are located on the cell surface of the endothelium of blood vessels and lymphatic vessels. Stimulation of these cells by VEGF leads to cellular growth, inhibition of cell death, and angiogenesis (Ferrara, Gerber et al. 2003). There are several isoforms of VEGF: 121-, 165-, 189-, and 206-amino-acid isoforms that can been formed by alternate splicing. VEGF acts in the regulation of both normal and abnormal angiogenesis, including neoplasm (Ferrara, Gerber et al. 2003). The VEGF is secreted by malignant neoplasm and by tumor-associated stromal cells (Fukumura, Xavier et al. 1998). mAbs, against VEGF signaling, induces growth inhibition of tumor (Kim, Li et al. 1993) (Wood, Bold et al. 2000). Bevacizumab, a humanized mAb against VEGF, was derived from murine mAb against human VEGF165 by Genentech. Bevacizumab has human IgG1 and kappa constant regions as human, while retaining murine complementarity determining regions (Presta, Chen et al. 1997). Bevacizumab reacts and neutralizes all isoforms of VEGF effectively (Kim, Li et al. 1992). Bevacizumab binds an epitope of VEGF that is distinct from the receptor-binding site (Liang, Wu et al. 2006). In a xenograft model with human rhabdomyosarcoma and breast carcinoma, bevacizumab inhibits tumor growth to under 10% of the original tumor weight and impaired tumor vasculature, decreasing microvessel density and permeability (Presta, Chen et al. 1997) (Salnikov, Heldin et al. 2006). The FDA approved bevacizumab for the first-line treatment of metastatic colorectal carcinoma in 2004. Recently FDA approved new indications including the use of bevacizumab in combination with 5fluorouracil based chemotherapy for first or second line treatment of patients with metastatic carcinoma of the colon or rectum. It is also used in combination with carboplatin and paclitaxel for the first line of treatment for advanced, recurrent, or metastatic nonsquamous cell, non small cell lung carcinoma.

2 GLIOBLASTOMA

2.1 Glioblastoma features

Malignant gliomas are tumors that develop in the central nervous system CNS, derived from the glial cells. Gliomas comprise a group of heterogeneous tumors classified for difference of cell type origin and histological features and are divided in astrocytoma, oligodendroglioma, oligoastrocytoma, ependymoma and choroid plexus tumors (Louis, Ohgaki et al. 2007). The astrocytomas account 75% of all gliomas. They were classified from the WHO into four prognostic grade for their typical characteristics as nuclear atypia, mitotic activity, endothelial proliferation and necrosis (Louis, Ohgaki et al. 2007) (Dolecek, Propp et al. 2012) and comprise: pilocytic astrocytoma (grade I), diffuse or fibrillary astrocytoma (grade II), anaplastic astrocytoma (Grade III), and glioblastoma multiforme (grade IV). The grade I and II are low grade malignancy, while those of III and IV grade are considered to be high grade malignant (Behin, Hoang-Xuan et al. 2003).
Glioblastoma multiforme (GBM) is the most frequent and the most aggressive malignant brain tumor. GBM derived from astrocytes cells, star shaped cells that support nerve cells, but can be composed from different cells (astrocytes and oligodendrocytes). Sometimes, they evolve from a low-grade astrocytoma or from an oligodendroglioma. In adults, GBM occurs most often in the cerebral hemispheres, especially in the frontal and temporal lobes of the brain. GBM is characterized for the worst survival rates with a median survival ranging from nine to twelve months after the diagnosis (Louis, Ohgaki et al. 2007). The standard therapeutic application, chemotherapy or radiotherapy, and the surgical resection do not improve the patient outcome. The term glioblastoma "multiforme" indicates the heterogeneous morphology, in which the cellular composition is highly variable. GBM is characterized by high genomic instability, uncontrolled cell proliferation, cells dedifferentiation, diffused infiltration, propensity for necrosis, strong angiogenesis and resistance to apoptosis (Furnari, Fenton et al. 2007; Shiras, Chettiar et al. 2007; Bonavia, Inda et al. 2011). This complexity combined with the presence of the putative CSCs and the epigenetic lesions makes the GBM one of the most difficult cancer to approach. GBM are subdivided into two subgroups: primary (or the novo GBM) and secondary GBM. The first one account in older patients and with any evidence of a prior symptoms or antecedent low grade, with a short clinical history. The secondary GBM, that represents a lower amount of the GBM cases, occurs in younger patients (less than 45 yr), and derives from a progressive transformation of low grade astrocytomas, generally grade II, that give arise to the high

grade. Primary and secondary GBM are morphologically and clinically identical although recent genomic profiles have revealed different transcriptional patterns and recurrent DNA copy number aberrations between primary and secondary GBM as well as new disease subclasses within each category (Maher, Brennan et al. 2006).

2.2 Signalling Pathways in Glioblastoma multiforme

Even if the primary and secondary glioblastoma multiforme are heterogeneous, they share specific cellular pathways of proliferation, survival, differentiation, migration, DNA repair, necrosis and apoptosis. These pathways include p53, pRB, growth factors, PI3K/Akt, apoptosis and angiogenesis signaling (Bonavia, Inda et al. 2011) (Fig 2).

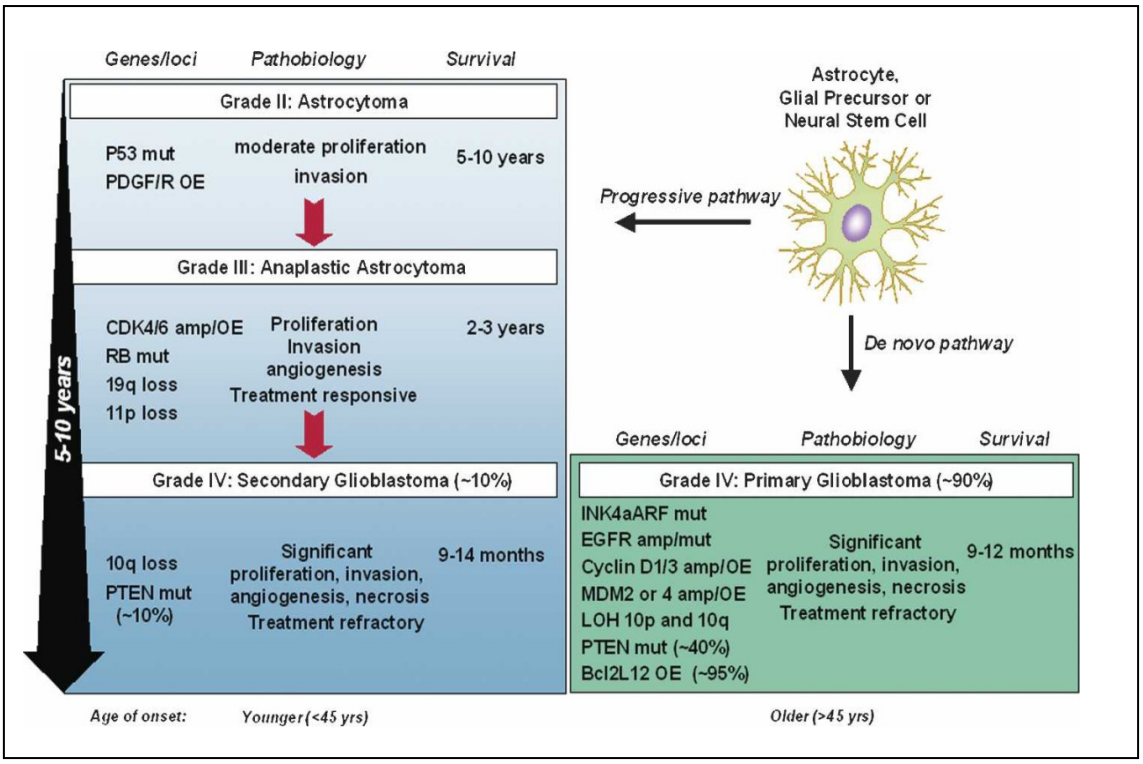


Fig 2: GBM can develop over 5–10 years from a low-grade astrocytoma (secondary GBM), or it can occur de novo (primary GBM). (Furnari et al, 2007)

Cell cycle gene disregulation in gliomas

Many mutations in gliomas account for cell cycle genes involved in cellular proliferation and senescence. The RB and p53 pathways are often targets of inactivating mutations in GBM.

RB pathway

The retinoblastoma protein (pRB) is a tumor suppressor that regulates the transition from G1 into S phase of cell cycle. The hypophosphorylated RB, in quiescent cells, sequester the E2F transcription factor preventing the activation of genes involved in the cell cycle progression (Furnari, Fenton et al. 2007). The mitogenic stimulation leads to the activation of the CDK complexes, in particular to the cyclin D1 activation that associated with the cyclin dependent kinase CDK4 and CDK6 phosphorylate RB leaving the E2F to promote the activation of genes to entry in S-phase and the DNA synthesis . The $p16^{INK4a}$ protein inhibits cell cycle progression negatively regulating the formation of the complex cyclin D/CDK (Sherr and McCormick 2002). In gliomas are frequent several alterations or amplifications in these genes. RB1 gene, localized in the chromosome 13q14, is mutated in around 25% of high grade astrocytomas. The 13q is loss in 50% of astrocytomas patients and the loss contributes to the formation of high-grade astrocytomas underlying that Rb inactivation is a tumor suppressor gene in astrocytoma tumorigenesis (Weinberg 1995). Amplification of CDK4 and CDK6 account in 15% of high gliomas determinate the functional inactivation of RB (Serrano, Hannon et al. 1993; Henson, Schnitker et al. 1994). Inactivating mutation by allelic loss or hypermethylation of $p16^{INK4a}$ is also account in 50% - 70% of high grade gliomas bringing to the lost of RB activity (Costello, Berger et al. 1996).

P53 pathway

The p53 (localized in chromosome 17p) gene codify for a tumor suppressor protein containing transcriptional activation, DNA binding and oligomerization domains. Cellular stresses as UV radiation, hypoxia, DNA damage and inappropriate oncogene activation activate p53 response, where p53 acts as a transcription factor promoting the expression of genes that block the cell cycle, as CDNK1A that encodes the protein inhibitor p21. p21 protein (WAF1) acts as inhibitor of CDK2, that is fundamental for the cycle progression in G1-S phase, then the cells blocked in G1-S phase can repair the DNA. If the damage is

irreparable p53 actives genes trigger apoptotic pathway, promoting cell death. Mdm2 negatively regulated p53 by ubiquitination and subsequent proteasomal degradation. P53 is also regulated by $p14^{ARF}$protein (localized in CDKN2A), that inhibits Mdm2, thus promoting p53 activation. p53 point mutations that inactive the DNA binding capacity or by loss of chromosome 17p are frequent in secondary GBM and in the progression of secondary glioblastoma (Louis 1994). The 2q14-15 region containing Mdm2 is overexpressed in some GBM and the corresponding gene $p14^{ARF}$is methylated so that its expression is inhibited in malignant gliomas. All these mutations determinate a propagation of cells with genomic instability (Louis 1994).

Mitogenic signaling pathway

The proliferation of normal cells requires the activation of signaling pathways triggered between extracellular matrix (ECM), growth factors, cytokines, cell-cell adhesion and their specific transmembrane receptors. These signals are transduced in the intracellular compartment by PI3K and MAPK signaling. In gliomas these pathways are constitutively activated.

PI3K/AKT/PTEN signaling

PI3K, phosphatidylinositide 3 kinases, is a family of enzymes divided in three classes (I,II,III), that phosphorylates membrane lipids, as phosphoinositides and phosphatidylinositols. The class I is formed by phosphatidylinositol- 4,5-bisphosphate (PtdIns(4,5)P2) and phosphatidylinositol 3,4,5-triphosphate (PtdIns (3,4,5) P3). PI3K can be activated after RTK (receptor tyrosine kinase) or integrin signaling.
PI3-kinases have been associated to a diverse group of cellular functions, including cell growth, proliferation, differentiation, motility, survival and intracellular trafficking. Many of these functions are related to the ability of class I PI3-kinases to activate protein kinase B (PKB, aka Akt) as in the PI3K/AKT/mTOR pathway. The class I is formed by two subunits: the p110 catalityc, also divided in three variants p110α, β, or δ, encoded by the PIK3CA, PIK3CB, and PIK3CD genes, and the p85 regulatory subunits, divided in five variants p85α, p55α, p50α, encoded by (PIK3R1) genes and p85β by (PIKR2), and p85γ by (PIKR3). In gliomas 15% frequency of mutation PIK3CA has been observed (Samuels, Wang et al. 2004) and PIK3D gene overexpressed in GBM (Gallia, Rand et al. 2006). The PtdIns (3,4,5)

P3 binds to different cytosolic proteins, including Akt which undergoes a conformational change that facilitates its activation through phosphorylation in correspondence of the two amino acid residues T308 and S473 by respectively phosphoinositide-dependent kinases (PDK) 1 and by the phosphatidylinositol 3-kinase-related kinase protein, the mammalian target of rapamycin mTOR, acting in the rapamycin-insensitive TORC2 complex (Mora, Komander et al. 2004). Akt is a key regulator of cell proliferation and survival pathways. It is a Serine/Threonine kinases able to inhibit apoptosis, promote cell proliferation and regulate lipids and glucose metabolisms, cell movements and vesicle trafficking. Akt is also inactivated by PHLPP (PH domain leucine-rich repeat protein phosphatase), which dephosphorylates S473, that was found to be expressed at very low levels in certain GBM cell lines and enhancing the anti-apoptotic activity of AKT (Maira, Galetic et al. 2001). The class I PI3K enzymes is antagonized by the PtdIns(3,4,5) P3 -phosphatase encoded by the PTEN gene. PTEN is a tumor suppressor that is inactivated in 50% of high grade gliomas by mutations or epigenetic mechanisms determinating the activation of uncontrolled PI3K activities. Deletions or mutations in the PTEN gene and high levels of phosphorylated or activated Akt are the most widespread alterations of the PI3K/Akt signaling in glioblastomas. These changes characterize GBM features, such as rapid tumor growth, invasiveness, resistance to cytotoxic treatments and massive angiogenesis (Knobbe, Trampe-Kieslich et al. 2005) (Rich and Bigner 2004) (Chakravarti, Zhai et al. 2004).

RTKs signaling

The receptors tyrosine kinase are transmembrane protein that share three basic components: the extracellular ligand binding domain, the transmembrane segment and an intracellular protein tyrosine kinase (PTK) domain with a catalytic core and the regulatory sequences. Upon binding of the ligand the receptor changes the inactive monomers into homodimers that activates its intracellular tyrosine kinase domain and autophosphorylate its C-terminal tyrosine residues. This stabilizes the receptor conformation and creates phosphotyrosine-docking sites for adapter proteins that transduce signals within the cell. Among the RTKs there are the Epidermal Growth Factor Receptor (EGFR) and platelet-derived growth factor (PDGFR). In gliomas the alterations of RTK include the overexpression of the ligands (growth factors) or of the receptors that lead to an autocrine activation of the pathways and the mutation of the receptors that are constitutively activated in absence of the specific ligands.

Epidermal growth factor receptor is a cell surface receptor family containing four structurally related members (erb1/EGFR, erb2/HER2, erb3/HER3 and erb4/HER4). Transduction by activated EGFR leads to downstream effect on proteins including phosphatidyl 3-kinase (PI3K), Phospholipase (PL) C-g1, Akt, Ras, Raf and mitogen-activated protein kinase (MAPK) that are associated with cell proliferation, motility and survival.

EGFR gene is amplified in around 40% of all GBMs, and the genes are frequently rearranged. In 20–30% of all human GBM occurs an EGFR mutant allele with deletion of exons 2–7 known as EGFRvIII (this mutant is also frequent in 50%–60% of those that have amplified wild-type EGFR), EGFR is constitutively actived and enhances tumorigenicity (Louis, Ohgaki et al. 2007). EGFRvIII is a validated target in gliomas and is reported that EGFR mutants have the capacity to enhance the proliferation and reducing the apoptosis and promoting the tumorigenesis in human (Frederick, Wang et al. 2000). EGFR is a target with crucial importance for the GBM therapy. There are small molecule kinase inhibitors, immunotherapy based on antibody, immunotoxins (Huang, Mukasa et al. 2007)and small interfering RNA against allele of EGFRvIII (Jungbluth, Stockert et al. 2003).

The PDGF signalling network consists of four ligands, PDGFA-D, the AB heterodimer ligand, and two receptors, PDGFR alpha and PDGFR beta. The alpha type binds to PDGF-AA, PDGF-BB and PDGF-AB, whereas the beta PDGFR type binds with high affinity to PDGF-BB and PDGF-AB. PDGFR pathway plays a role in embryonic development, cell proliferation, cell migration and angiogenesis.

PDGF-A and PDGF-B are expressed in highgrade glioblastoma and strong expression of PDGFR occurs in endothelial cells in GBM (Fan and Weiss 2005). PDGF-C and PDGF-D are also often expressed in glioma cell lines and in GBM tissues (Westermark, Heldin et al. 1995). The amplification or the rearrangement of PDGFR is much less common but is reported a loss of exons 8 and 9 of PDGFR that determinate a constitutive activation of the receptor enhancing the tumorigenicity (Lokker, Sullivan et al. 2002).

Apoptosis

The main feature of glioma cells is its resistance to chemotherapy and to radiotherapy treatment. This property is due to genetic alterations of regulatory molecules involved in mitogenic and/or PI3K/PTEN/Akt pathways and/or in classical cell death networks of extrinsic (death receptor-mediated) and intrinsic (mitochondria-dependent) apoptosis signaling (Clarke and Dirks 2003).

The death receptors are cell surface proteins that after binding of cognate ligands, recruit adapter molecules to create a protein complex that has an autoproteolytic capacity determinating the activation of caspases. The most important system of death receptor include TNFR1, TRAILR1-2 and CD95. These death receptors are often down-regulated and their ligand mutated in glioma pathogenesis (Maher, Brennan et al. 2006).

It was reported that human glioma cell lines and primary glioma-derived cell cultures are sensitive to death ligand-mediated apoptosis in vitro and in vivo (Igney and Krammer 2002). Expression levels of death receptors and of their decoy receptors (antagonistic) were associated with vulnerability of glioma cells to death ligand-induced apoptosis. The expression of antagonist receptor for CD95 ligand (CD95L), the soluble decoy receptor 3 (DcR3), on malignant glioma cell lines was correlated with the grade of malignancy in human glioma specimens. Even more, the infiltration of CD4+ and CD8+ T cells and microglia/macrophages was significantly decreased in DcR3-driven xenografts, suggesting that a decoy receptor neutralizes CD95L attack by preventing its interaction with the receptor (Maleniak, Darling et al. 2001).

The expression of TRAIL death receptor system has been correlated with survival of patients with primary GBM (Roth, Isenmann et al. 2001). The treatment with TRAIL inhibited growth of human glioma cell xenografts (Kuijlen, Mooij et al. 2006) and acted synergistically with chemotherapeutic drugs (Roth, Isenmann et al. 1999), determinating a up-regulation of TRAIL-R2 and Bak protein and down-regulation of the cFLIPs (caspase-8-specific inhibitor) (Nagane, Pan et al. 2000) . Peptides as Smac, a potent antagonist of the IAP family (Inhibitors of Apoptosis Protein), shown a synergistic effect with TRAIL to induce tumor cell apoptosis in vitro and in vivo without demonstrable neurotoxicity (Song, Song et al. 2003) underling the importance of post-mitochondrial caspase activation for apoptosis propagation in glioma cell lines and its validity as a therapeutic target (Song, Song et al. 2003).

Bcl-2 family is composed by pro-apoptotic (Bax, BAD, Bak) or anti-apoptotic (including Bcl-2 proper, Bcl-xL, and Bcl-w, MCL-1) proteins. These proteins control the mitochondrial membrane integrity and the release of cytochrome c, which effects the caspase cascade and the apoptotic program (Fulda, Wick et al. 2002). There is a correlation between tumor grade and expression of anti-apoptotic Bcl-2 proteins (BCL-2 and MCL-1) (Green and Kroemer 2004). For example Bcl-xL was found up-regulated by overexpression of EGFRvIII in glioma cells and this upregulation confers resistance to the chemotherapeutic agent as cisplatin (Krajewski, Krajewska et al. 1997). Bcl2 family may also contribute to gliomagenesis through enhancement of migration and invasion by altering the expression of metaloproteinases and their inhibitors (Wick, Wagner et al. 1998; Wick, Grimmel et al. 2001).

Necrosis

GBM tumor cells are resistant to therapeutic apoptotic stimuli but show wide cellular necrosis. Necrosis in fact is the major form of spontaneous cell death in GBM (Wick, Wild-Bode et al. 2004). Necrosis is determinated by inadequate blood supply and anoxia due to a microthrombotic process. In this regard was found a new protein Bcl2-like 12 (Bcl2L12) protein, an inhibitor of post-mitochondrial apoptosis signal transduction, that is significantly overexpressed in primary GBMs (Brat and Van Meir 2004). Bcl2L12 is a protein with homology with the BH (Bcl-2 Homology) 2 domain found in several members of the Bcl-2 protein family (Stegh, Kim et al. 2007). Overexpression of Bcl2L12 in primary astrocytes inhibited apoptosis, and its RNAi-mediated knockdown sensitizes human glioma cell lines to drug-induced apoptosis and reduces tumor formation in an orthotopic model system. Bcl2L12 protein neutralizes effector caspase activity determinating an anti-apoptotic effect (Brat and Van Meir 2004). The activities of Bcl2L12 explain the necrotic process in which the suppression of caspase activity from mitochondria redirects the death program from apoptosis to necrosis (Scorilas, Kyriakopoulou et al. 2001).The interconnection between apoptosis and necrosis signaling and Bcl2L12 is represented by the Bcl2L12 expression that induces necrotic cell morphology, as evidenced by substantial plasma membrane disintegration and enhanced nuclear and subcellular organelle swelling in apoptosis-primed astrocytes (Brat and Van Meir 2004).

Angiogenesis

GBMs are the most highly vascular solid tumors. Microvascular hyperplasia consists of endothelial cells in proliferative activity that come out from normal parent microvessels as microaggregates (glomeruloid bodies) accompanied by stromal elements, including pericytes and basal lamina (Nicotera and Melino 2004). Microvascular proliferation is a features that characterize the evolution from low-grade or anaplastic astrocytomas to secondary GBM (Maher, Brennan et al. 2006) . In the last decade has been reported that tumor-associated angiogenesis is not due only to physiological modification to hypoxia resulting from an increase of tumor cell mass but it appears to be the result of genetic mutations that activate a transcriptional program for angiogenesis with local tumor oxygen status modifying this response. For example mutations, including those in the PTEN, EGFR, and CMYC genes, may act as an "angiogenic switch" by stabilizing HIF-1 (Hypoxia inducing factor) or one of its downstream targets, VEGF (Stiver, Tan et al. 2004) (Watnick, Cheng et al. 2003) (Blum, Jacob-Hirsch et al. 2005) (Phung, Ziv et al. 2006).

Tumor cell invasion

The brain glioma's invasiveness is the most important characteristic of low- and high-grade malignant glioma and is the cause of the failure of surgical cure. In most of the cases the tumor develops adjacently to the resection margin. Invasion is determinated by processes that involve cell interactions with the ECM and with adjacent cells and by biochemical proteolytic degradation of ECM and active cell movement.
Glioma cells invade along the white matter tracts and the basement membranes of blood vessels, accessing to a disrupted blood vessel within the tumor. Gliomas are unable to establish robust tumor growth outside the CNS and is unable to metastasize outside the CNS. The invasion and migration of glioma cells in fact differs from other tumors where local spread is very limited and dissemination occurs hematogenously or via the lymphatic system (Lefranc, Brotchi et al. 2005). Several genes have been identified in glioma invasiveness and include the family of metalloproteases (MMP) and their endogenous tissue inhibitors (TIMPs). Expression of MMP-2 and MMP-9 are associated with invasiveness, proliferation and prognosis in astrocytomas (Wang, Wang et al. 2003). Other non-MMP proteases, including urokinase-type plasminogen activator (uPA) (Landau, Kwaan et al. 1994) and cysteine proteases, as cathepsin B, are elevated in high-grade malignant gliomas

(McCormick 1993). The role of proteases in glioma invasion is unclear because low-grade astrocytomas infiltrate throughout the brain, despite relatively normal levels of the proteases. Also integrins, as αVβ3 complexes, are overexpressed in GBM and are important in glioma invasion and angiogenesis (Uhm, Dooley et al. 1997) (Kanamori, Vanden Berg et al. 2004). Other proteins are overexpressed in invasive areas of GBM, such as angiopoietin- 2, the ligand of Tie receptors, which is involved in angiogenesis and determinate infiltration by activating MMP-2 (Hu, Guo et al. 2003).

Ephrin receptors and their ligands, the ephrins, have been shown to control migration and invasion. EphA2 and EphB2overexpression has been associated to poor survival in GBM (Liu, Park et al. 2006) .

2.3 Glioblastoma treatment

Glioblastomas is the most difficult cancers to treat for its complexity for the drug distribution within the intracranial space, due to the presence of the blood-brain barrier. GBM grow quickly and show a high resistance to apoptosis (Rich and Bigner 2004; Wen and Kesari 2008).

The standard therapy for malignant gliomas involves surgical resection, when feasible, radiotherapy, and chemotherapy. GBM is still associated with poor prognosis with the median survival of 1 year (Wen and Kesari 2008). Malignant gliomas cannot be completely eliminated surgically for their infiltrative nature, but patients undergo to maximal surgical resection whenever possible. Advances in MRI (magnetic resonance imaging) have improved the safety of surgery and increased the extent of resection that can be achieved. Radiotherapy is the primary intervention of treatment for malignant gliomas. The addition of radiotherapy to surgery increases survival among patients with glioblastomas from a range of 3 to 4 months to a range of 7 to 12 months (Walker, Alexander et al. 1978). Recently has been reported that chemotherapeutic agents may improve the efficacy of radiotherapy, resulting in a modest increase in survival (a 6 to 10% increase in the 1-year survival rate) (Fine, Dear et al. 1993; Stewart 2002). In the last years, the combination of radiotherapy and temozolomide (TMZ), an oral alkylating agent with good penetration of the blood brain barrier, shown an increasing in the median survival (14.6 months vs 12.1 months, $p<0.001$) (Stupp, Mason et al. 2005). Radiotherapy with concomitant adjuvant temozolomide is a useful combination for newly diagnosed glioblastomas. Others agents used reflects the prevalence of alterations in EGFR, such as the EGFRvIII deletion mutation (Scott, Lee et al. 2007) and PDGF signaling and modulators of PI3K signaling, as well as the prominence of biological processes such as angiogenesis and invasion. Research is focusing also on others molecules against the integrins as Etaracizumab, CNTO95, Cilengitide, Volociximab. Vitaxin, the precursor of etaracizumab, is used in Phase I trials showed antiangiogenic activity and a Phase II study showed some efficacy in metastatic melanoma. CNTO95, which targets both $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, also had anti-tumour and anti-angiogenic effects in xenograft tumour models. Cilengitide is currently being tested in Phase II trials in patients with lung and prostate cancer, and Phase II and Phase III trials are currently underway in glioblastoma. In the same time the integrins are targets for cancer imaging and drug discovery. Integrins have the ability to deliver diagnostic agents (radionuclides, Cu, F, superparamagnetic iron oxidase suitable in

Scintigraphic or PET) and chemoterapeutics or proapoptotic peptides to tumour cells (Integrin αvβ3 targeted nanoparticles to delivery mutat RAF1 to the tumor vasculature, resulting in apoptosis of endothelial cells and tumor regression or with nanoparticles loaded with doxorubicin) (Desgrosellier and Cheresh 2010) (Guo and Giancotti 2004).

3 GLIOBLASTOMA STEM CELLS

3.1 Glioblastoma stem cells features

Accumulating literature, from almost 20 years of research, support the hypothesis of cancer stem cells and the importance to identify them with specific markers.
This hypothesis was initially demonstrated in blood tumors where in acute myeloid leukemia a subpopulation of leukemic cells (CD34+/CD38-) maintain the ability to propagate the tumor after serial transplantations in recipient immunodeficient mice models, reproducing a tumor with the same characteristic of the original (Bonnet and Dick 1997). The demonstration that, as in hematological malignancies, solid tumors arise from a cancer stem cell (CSC) opens a new scenario for understanding the biology of the tumor. These studies suggest the existence of a subpopulation of cells, the cancer stem cells within the tumor mass (about 2% of the total cancer cell population) with a crucial role in the development of the tumor. This model of cancer growth is called hierarchical model (or cancer stem cell hypothesis). This model implies that the cancer stem cells are at the apex of the hierarchy and for their stemness proprierties, as self renew and the capability to give arise to progenitors cells, are responsible for the origin and for maintenance of cancer (Maugeri-Sacca, Bartucci et al. 2012).The bulk of cancer cells within a tumor are progeny of CSCs, have not tumorigenic potential, thus cannot regenerate new tumors, and might represent a mix of partially differentiated cancer progenitor like cells with limited proliferative capacity, terminally differentiated, an death committed cancer cells (Tang, Ang et al. 2007). On the other site the stochastic clonal model affirm that random occurrence of mutations lead to dominant clones that gain a survival advantage over other cells, with more ability to thrive in a hostile microenvironment and to adapt to microenvironmental perturbations. More recently is been suggested a new model “clonal- hiercharchical model” within the stem cells population. These model proposed the evidence that are reported in different works. Infact the stemness features has been found to be induced in differentiated cells upon forced expression of embryonic stem cell-specific transcription factors (Takahashi and Yamanaka 2006), or by exogenous influences as hypoxia, low pH, signals as stimulation with hepatocyte growth factor, and activation of the epithelial-mesenchymaltransition (EMT) program (Mani, Guo et al. 2008) (Li, Bao et al. 2009) are involved in the process to maintain and to enrich CSCs. This underline that the retention/acquisition of stem-like features is a dynamic process (Fig 3).

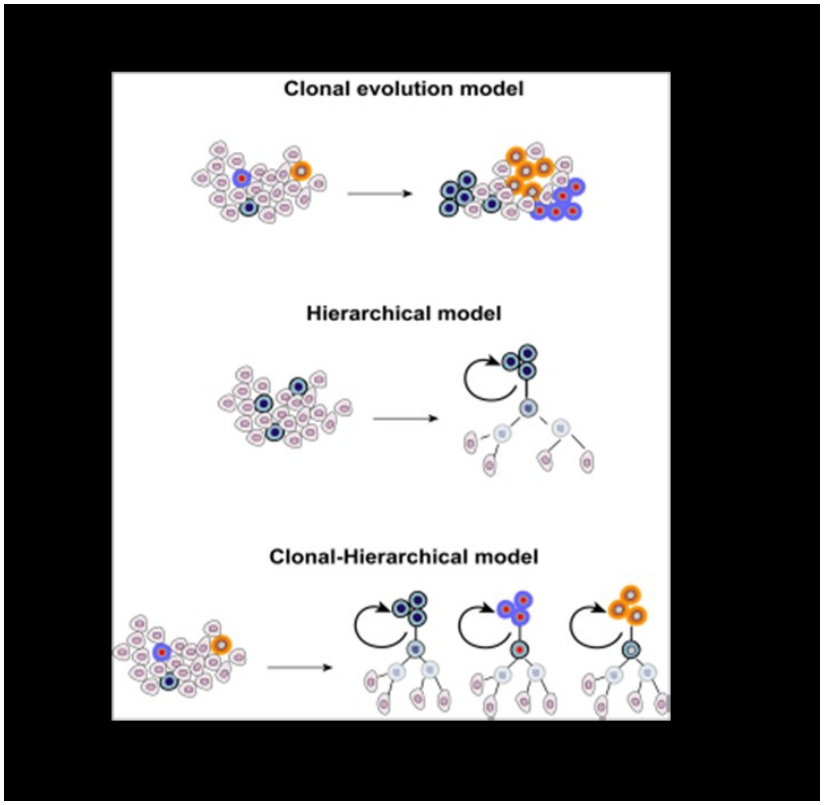


Fig 1 Different models to explain the tumor evolution
Clonal evolution model) Different clones in the tumor have the same ability to proliferate and to retain tumorigenicity, and the random genetic mutations confer dominant traits to some of them; Hierarchical model) A stem-like cell located at the apex of the tumor pyramid is the precursor of the whole tumor population ;Clonal Hierarchical model) the combined clonal-stemc ell model suggests that CSCs can undergo clonal evolution and multiple CSC clones coexist within the tumor. (Maugeri-Saccà 2013)

CSCs, in solid tumor, were first characterized by Al-Hajj and coworkers (Al-Hajj, Wicha et al. 2003), who isolated mammary stem cells (CD44+CD24-) from human breast carcinoma. After many groups attempted *in vitro/in vivo* characterizations of populations enriched in CSCs isolated from solid tumors.

CD133, also called Prominin-1, a cell surface glycoprotein, localizes on membrane which suggests an involvement in the mechanisms influencing cell polarity, migration and interaction of stem cells with neighbouring cells (Dell'Albani 2008).

CD133 was shown to be expressed on normal human neural stem cells (Uchida, Buck et al. 2000) and was been found on CSC cells isolated from various compartment (Wu and Wu 2009). CD133+ cells isolated and transplanted into NOD/SCID mice recapitulated the heterogeneity of the original tumor. Dirks group in 2004 demonstrate that CD133 positive cell subpopulation from human brain tumors exhibited stem cell properties in vitro and in vivo assay (Singh, Clarke et al. 2003). Recently CD133, as cancer stem cell markers, was in the middle of a debate whereas CD133- cells can shared the same properties of CD133+ cells (Chen, Li et al. 2010).

New markers have been used to identify specifically and target GSCs (Glioblastoma stem cells): SSEA-1, L1CAM, A2B5, Side Population, Nestin, OCT-4, ALDH, Integrinα6 (table 1).

Parameter	Assessment	Functional correlate	Key reference
CD 133	Magnetic sorting or flow cytometry sorting for CD133 expression	Self renewal Multipotency Tumorigenicity *in vivo*	Singh et al. 2004
Alpha 6 integrin	Sorting for alpha 6 integrin expression alone or in combination with CD133 expression by flow cytometry	High enrichment for cells with self-renewal Sphere formation Tumorigenicity *in vivo*	Lathia et al. 2010
Culture condition	Culture condition: serum-free medium supplemented with EGF, FGF	Self renewal Multipotency Tumorigenicity *in vivo*	Galli et al. 2004 Günther et al. 2008
Side population	Flow cytometry sorting based on exclusion of Hoechst 33342 (side population)	Tumorigenicity *in vivo* only in side population	Kondo et al. 2004
A2B5	Flow cytometry sorting for A2B5 and CD133 expression	Tumorigenicity	Ogden et al. 2008
ALDH1 activity	Flow cytometry sorting for ALDH1 activity	High ALDH1 levels keep glioma cells in an undifferentiated state High ALDH1-expressing glioma cells grow in neurospheres ALDH1 inhibition induces differentiation and reduces clonogenicity	Rasper et al. 2010
Autofluorescence	Intrinsic autofluorescence and morphology	Self renewal Multipotency Tumorigenicity *in vivo*	Clement et al. 2010

Table I **Cell surface markers and functional assays for the isolation of glioma stem-like cells (Ghazaleh Tabatabai & Michael Weller, 2011)**

SSEA-, also called CD15, is a stage-specific embryonic antigen 1 and was shown that the selection for SSEA-1+ cells enriched for glioma tumor stem cells (TSCs) or tumor-initiating cells (TICs). These SSEA-1+ cells give at least a 100-fold tumorigenic enrichment in mouse xenograft models (Son, Woolard et al. 2009).
L1CAM , called L1 or CD171, has been identified as a potential therapeutic target in neuro-oncology. L1CAM regulates neural cell growth, survival, migration, and axonal outgrowth and neurite extension during central nervous system development. Although the role of L1CAM in the normal adult nervous system is not well defined, L1CAM is overexpressed in gliomas and other solid cancers, including colorectal cancer where L1CAM is used as prognostic indicator (Bao, Wu et al. 2008).
A2B5 is a glial progenitor marker and the authors identified a population of A2B5+ CD133- cells, phenotypically distinct from CD133+ cells, showing a tumorigenic properties (Ogden, Waziri et al. 2008).

A common way to identify CSCs is by exclusion staining with Hoechst 33342 dye. A main feature of CSCs and normal stem cells is the high expression levels of the ATP-binding cassette (ABC) transporters that are responsible for multidrug resistance and able to export the dye. This identify an unlabelled "side population" (SP) highly enriched in stem cells in many tissues, including neural (Kim and Morshead 2003). SP fraction isolated from the rat glioma cell line C6 show stem cell-like properties. The same results were observed for human glioma cell lines. However, there are conflicting data showing that either the sorted SP or non-SP cells were similarly clonogenic in vitro and equally tumorigenic in vivo.

Another approach to select the glioblastoma stem cells is the autofluorescence emission from the cells. This was used to enrich a subpopulation with self-renewal ability in vitro, tumor initiating and propagating capacity in vivo (insert Clement et al, 2010). In addition, high aldehyde dehydrogenase (ALDH) activity has been used as a functional marker to isolate CSCs. These enzymes detoxify aldehydic products generated by reactive oxygen species and might therefore participate in cell survival. High ALDH1 activity keep glioma cells in an undifferentiated state. It is reported that ALDH1 inhibition induces differentiation and reduces clonogenicity in vitro (Rasper, Schafer et al. 2010).

Recently Integrin α6 was suggested as new marker for glioblastoma stem cells. Sorting for integrin α6 high alone or in combination with CD133 led to enrichment of cells that display GSC properties. Integrin α6 depletion using short hairpin RNA or treatment with integrin-

blocking antibody determinated to growth inhibition in vitro and reduced tumor formation in vivo (Lathia, Gallagher et al. 2010).

Nestin is an intermediate filament (IF) protein expressed in the stem and progenitor proliferating cells during the central nervous system developmental stages and its expression is down-regulated in differentiated cells. It is involved in the organization of the cytoskeleton, cell signaling and metabolism, organogenesis, and represents the proliferation, migration and multi-differentiated characteristics of multi-lineage progenitor cells (Hadjipanayis and Van Meir 2009).

3.2 Altereted pathways in Glioblastoma stem cells

The Glioblastoma stem cells differ from normal neural stem cells for a deregulation of the signal involved in fundamental cellular processes such as cell proliferation, differentiation, stemness maintenance, drug and radio resistance (Li, Wang et al. 2009). The main pathways altered in GSCs include Notch, Hedgehog-GLIs, growth factors, BMP, and TGF signalling.

Notch pathway

Notch proteins are a family of single pass transmembrane domain receptors involved in cell-cell communication. After the binding of its ligands (Jagged 1-2, Delta like 1-3-4), Notch is cleaved by the γ-secretase complex. The Notch intracellular domain is thus released from the plasma membrane, and translocates into the nucleus where it acts as a transcription factor. During development, Notch promotes the proliferation of normal NSCs while suppressing their differentiation. Notch functions were linked to glioma CSCs, as Notch signaling increases expression of the stem cell marker as Nestin. Moreover, activation of Notch signaling in the glioma cell lines increases the formation of neurosphere-like colonies (Huang, Cheng et al. 2010) (Zhang, Zheng et al. 2008).

Hedgehog pathway

The binding of Hedgehog ligands to their receptors activates transducers termed GLIs (named for their discovery in gliomas), which then translocate into the nucleus to activate or repress downstream targets. The Hedgehog pathway is a regulator of embryogenesis, to transmits information to embryonic cells required for proper development, and is involving different types of normal stem cells, including NSCs (Wechsler-Reya and Scott 1999). Hedgehog signaling is also active in gliomas and contributes to GSCs function, favoring cell proliferation, self-renewal, tumorigenicity and drug resistance (Clement, Sanchez et al. 2007). Furthermore, it has been demonstrated that Hedgehog inhibitors, as cyclopamine, improve traditional therapy efficiency against gliomas. In particular, Bar and colleagues demonstrated that cyclopamine treatment improves the effects of radiation on GSC survival (Bar, Chaudhry et al. 2007).

Growth factors signaling

GSCs are also characterized by the deregulation of growth factor signalling (EGF, bFGF, PDGF and IGF). GSCs activate these pathways through different mechanisms, such as ligand and/or receptor overexpression, receptor mutation causing its constitutive activation, intracellular messenger activation through mutation or the loss of expression of negative regulators (Huang, Cheng et al. 2010). The signal initiated by the binding between growth factors and their receptors (Receptor Tyrosine Kinases, RTKs) is transduced and amplified through downstream molecule cascades, such as the pro-survival AKT/PI3K pathway. Upon activation, AKT promotes survival, proliferation, invasion, and secretion of proangiogenic factors. It has been recently demonstrated that GSCs are more dependent on AKT signals than non-stem glioma cells. Pharmacologic inhibitors of AKT attenuate the generation of neurospheres, the structures usually formed by GSCs in culture, suggesting that AKT inhibition may specifically target the GSC population to reduce tumor malignancy (Eyler, Foo et al. 2008).

BMP pathway

Bone Morphogenic Proteins (BMPs) are a family of growth factors involved in bone and cartilage development. Upon the binding to the cell-surface receptor kinases, the BMPRs, Smad1/5/8 proteins are activated and bind to the co-activator Smad4 that translocates into the nucleus and regulate transcription. BMPs regulate the proliferation, apoptosis and differentiation signal in neural stem cells (Li, Wang et al. 2009). It was shown that this pathway is altered in GSCs by epigenetic alterations of BMP receptor leading to a reduced expression and signal transduction; GSCs are able to escape the differentiation process induced by BMPs maintaining intact their stemness proprieties (Lee, Son et al. 2008).

TGF-β pathway

The TGF-β is a superfamily that includes proteins that regulate differentiation and cellular development processes (Huang, Cheng et al. 2010). It was shown that GSCs produce and release TGF-β into the extracellular microenvironment inducing cell survival and stemness maintenance through the activation of Sox2 signal transduction pathway (Penuelas, Anido et al. 2009).

Sox2-Oct4-Nanog

Many intracellular factors are considered important for the regulation of survival, proliferation and maintenance of stemness proprierties in gliomas cancer cells as Sox-2, Oct-4, Nanog, bm1, c-myc and mirRNA. Sox2, Oct4 and Nanog are transcription factors fundamental for the regulation between self-renewal and differentiation in embryonic and adult stem cells (Huang, Cheng et al. 2010). The Oct4 expression is correlated with the glioma grade and it is highly expressed in many human glioma specimens and cell lines. Oct4overexpression in rat glioma cells increases the expression level of the stemness marker Nestin suggesting that Oct4contribute to CSC stemness maintenance.

Olig2

Olig2 is a transcription factor expressed in the CNS (central nervous system). It is expressed in both normal neural stem cells and glioma cancer stem cells.
Olig2 is involved in the development of CNS and it is expressed in neural progenitor cells that give rise to oligodendrocytes and substains the multilineage differentiation potential of neural progenitors (Li, Wang et al. 2009). Olig2 was found to be expressed in almost all adult astrocytomas and is required for tumor initiation, suggesting a link to gliomas and CSCs (Ligon, K. L et al. 2004) (Ligon, K. L.et al 2007).
In GSCs Olig2 is involved in the inhibition of the differentiation process through the suppression of the cell cycle regulatory protein p21*WAF1/CIP1* (Ligon, K. L.et al 2007).

c-Myc

c-Myc is a transcription factor, considered an oncoprotein, that plays an important role in the proliferation of both normal stem cells and tumor cells. Recently, inducible pluripotent stem cells were generated from differentiated cells through the introduction of several transcription factors, including c-*myc* (Takahashi, Ichisaka et al. 2006), supporting a role in core stem cell machinery. c-Myc expression correlates with the grade of malignancy in gliomas (Herms, von Loewenich et al. 1999). This suggests a link between the stemness and malignancy. Glioma cancer stem cells express high levels of c-Myc and is required for maintenance of GSCs *in vitro* and for GSCs tumorigenic capacity *in vivo* (Zheng, Ying et al. 2008). It has been demonstrated that c-Myc additionally prevents differentiation and

promotes self-renewal of tumor neurospheres derived from a p53/PTEN double knock-out mouse model (Zheng, Ying et al. 2008).

BMI1

BMI1 belongs to the Polycomb group genes, that function as epigenetic silencers. BMI1 has been implicated in determining stem cell fate in multiple tissues and is a positive regulator of neural stem cell selfrenewal. BMI1 is also a known oncogene frequently overexpressed in many cancer types, including gliomas (Li, Wang et al. 2009). BMI1 is overexpressed also in GSCs and is required to sustain the self-renewal process of these cells (Abdouh, Facchino et al. 2009).

miRNAs

miRNAs are small noncoding RNAs that can silence target genes through post-transcriptional mechanisms on target mRNAs. A single miRNA can regulate many distinct mRNAs. In cancer biology, miRNAs can function as oncogenes or as tumor suppressors. miRNAs play an important role also in glioma CSCs. For example the levels of miR-124 and miR-137 are reduced in grade III and IV malignant gliomas in comparison with normal brain. Overexpression of these two miRNAs inhibits proliferation and induced differentiation of glioma CSCs, indicating a tumor suppressor role for these two miRNAs in GSCs (Silber, Lim et al. 2008). Similarly, another miRNA, miR-451, is expressed at lower levels in CD133+ GSCs in comparison with CD133− non-stem glioma cells. In particular, it has been demonstrated that miR-451 inhibits the growth of glioma CSCs and disrupts the formation of cancer stem neurospheres (Gal, Pandi et al. 2008).

Hypoxia

Brain Tumor Stem Cells (BTSCs), similar to their normal stem cell counterpart, are localized in specific microenvironments or stem cell niches that include hypoxic regions, the perivascular compartment and the invasive edge. CSCs, as NSCs, can recruit vessels during tumorigenesis and seem to have angiogenic properties. It was reported that the number of capillaries correlates with the GBM patients' prognosis (Leon SP et al, 1996). Glioma stem cells are located preferentially in the perivascular area, in fact endothelial cells were shown to interact with CD133+/Nestin+ cells isolated from glioblastoma, medulloblastoma,

ependymomas, and oligodendrogliomas and to produce factors that maintain these cells in a self-renewing and undifferentiated state. Co-injection of CSCs and endothelial cells into immunocompromised mice accelerates the initiation and the growth of brain tumors (Calabrese et al, 2007).

GBM cancer stem cells are regulated by microenvironment conditions such as hypoxia that stimulate the growth of the neovasculature by expressing high levels of pro-angiogenic factors such as VEGF (Bao S. et al, 2006a). On the other hand, blood vessels create a vascular niche to maintain CSC population. Endothelial cells express Notch ligands that may stimulate Notch receptors essential for CSCs maintenance (Fan et al, 2010) and may also secrete nitric oxide to activate the Notch pathway (Charles et al, 2010). CSCs are also regulated through ECM receptors, such as integrin α6, that are enriched on GBM CSCs and promote their maintenance (Lathia et al, 2010).

Hypoxia in neural tumors have been well characterized close to areas of necrosis. Low oxygen promotes the mantainance of an undifferentiated cell state in normal tissues and in tumors supporting a niche for CSCs. In hypoxic conditions, non-stem glioma cells acquire self-renewal and long-term proliferative potential, in addition to the expression of genes related to stem cell functions. Hypoxia modulates cell phenotypes via hypoxia-inducible factor (HIF) signaling which drives expression of stem cell-related genes. The expression of HIF2α is specific for CSCs and is associated with poor glioma patient survival.

3.3 Glioblastoma stem cells pathogenesis

GBM is one of the most lethal disease with a high rate of recurrence after treatment. In the last decade a lot of literature underlines that GSCs have a crucial role in the GBM malignant behavior, because are involved in the processes of radio and chemo-resistance, recurrence, metastasis and angiogenesis (Xie 2009), (Hadjipanayis and Van Meir 2009) (Salmaggi, Boiardi et al. 2006). GSCs appear to contribute to the radio-resistance. Bao and colleagues demonstrated that GSCs, in response to DNA damage induced by ionizing radiation rapidly activate the response to DNA damage by phosphorylation of proteins of cell cycle pathway as ATM,Rad17, Chk1 and Chk2 determinating cell survival. In particular, CD133-expressing tumor cells preferentially activate the DNA damage check points in response to radiation compared with the CD133-negative tumor cells (Bao, Wu et al. 2006). The GSCs are resistant also to cytotoxic drugs. This was to be linked to high expression levels of the DNA repair protein MGMT, that remove the methyl groups added to the DNA by alkylating agents. This underlines the resistance of GSCs to pharmacological treatment (Liu, Yuan et al. 2006). GSCs overexpress ABC transporter family (ABCG2 proteins) (Dean, Fojo et al. 2005). These proteins able to transport anticancer drugs outside the cells. This make them resistance to the treatment. It was shown that GSCs have alterations in the apoptotic signaling for their overexpression of apoptosis suppressor such as Bcl2, Bcl-XL, FLIP and several inhibitors of apoptosis, such as IAPs proteins which bind and inhibit caspases 3-7-9, preventing apoptosis (Johnstone, Ruefli et al. 2002; Liu, Yuan et al. 2006). GSCs are also considered responsible of the high angiogenesis that characterize the glioblastoma. Angiogenesis is a process to provide oxygen and nutrients to the tumor mass and also for metastasis. Bao et all demonstrated that GSCs promote angiogenesis amplifying the secretion of VEGF, one of the most important pro-angiogenic factor (Bao, Wu et al. 2006). Treatment with bevacizumab, antibody that neutralizes the effect of VEGF, shown a decrease of GSCs to support the angiogenesis. The upregulation of VEGF in GSCs is not clear but it's supposed that hypoxia could play an important role (Li, Wang et al. 2009).

4 INTEGRINS

The “integrins” include (Mitra and Schlaepfer 2006) a family of structurally, immunochemically, and functionally related cell-surface heterodimeric receptors, that is integrated between the extracellular matrix and the intracellular cytoskeleton to mediate cell migration and adhesion. The β subunits account to eight and the at 17 subunits. These subunits interact non covalently in a restricted manner to form more than 20 family members. The diversity of integrins is expanded further by alternative splicing, post-translational modifications, and interactions with other cell-surface and intracellular molecules (Guo and Giancotti 2004). A hallmark of the integrins is the ability of individual family members to recognize multiple ligands. Table 2 summarizes the major extracellular ligands of integrins. The list includes a large number of extracellular matrix proteins as bone matrix proteins, collagens, fibronectins, fibrinogen, laminins, thrombospondins, vitronectin, and von Willebrand factor, reflecting the primary function of integrins in cell adhesion to extracellular matrices. The preference of any given integrin among its ligands is determined by relative affinity, availability within a specific microenvironment, and the conformational state of the ligand, which controls exposure of its integrin recognition sequence.

Integrin biology

Integrins are clustered in the membrane and recruit various signalling and adaptor proteins to form structures known as focal adhesions. Although integrins lack kinase activity by them self, the clusters that are formed recruit and activate kinases, such as focal adhesion kinases (FAKs) and src family kinases (sFKs), in addition to scaffolding molecules, such as p130 CRK-associated substrate (p130CAs; also known as BCAR1). Integrins also couple the ECM to the actin cytoskeleton by recruiting proteins, including talin, paxillin, α-actinin, tensin and vinculin. Additionally, a ternary complex consisting of an integrin-linked kinase, PInCH (also known as lIMs1), and parvin regulates many scaffolding and signalling functions required for integrin-mediated effects on cell migration and survival (Legate, Montanez et al. 2006). Furthermore, integrin recruitment to membrane microdomains by tetraspanins might crucially regulate integrin function in tumor cells (Zoller 2009). Regulation of the recruitment and activation of these and other focal adhesion proteins influences cell adhesion and migration on the ECM. In fact, many of these molecules are themselves being investigated as possible targets for cancer therapy. In some cases, the function of an integrin is related to its ligand affinity. Increased affinity or activation can be

Integrin extracellular ligands

Ligand	Integrin
Adenovirus penton base protein	$\alpha_v\beta_3$, $\alpha_v\beta_5$
Bone sialoprotein	$\alpha_v\beta_3$, $\alpha_v\beta_5$
Borrelia burgdorferi	$\alpha_{IIb}\beta_3$
Candida albicans	$\alpha_M\beta_2$
Collagens	$\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{11}\beta_1$, $\alpha_{II}\beta_3$
Denatured collagen	$\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
Cytotactin/tenascin-C	$\alpha_8\beta_1$, $\alpha_9\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$
Decorsin	$\alpha_{IIb}\beta_3$
Disintegrins	$\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
E cadherin	$\alpha_E\beta_7$
Echovirus 1	$\alpha_2\beta_1$
Epiligrin	$\alpha_3\beta_1$
Factor X	$\alpha_M\beta_2$
Fibronectin	$\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_{IIb}\beta_3$
Fibrinogen	$\alpha_5\beta_1$, $\alpha_M\beta_2$, $\alpha_v\beta_3$, $\alpha_x\beta_2$, $\alpha_{IIb}\beta_3$
HIV Tat protein	$\alpha_v\beta_3$, $\alpha_v\beta_5$
iC3b	$\alpha_M\beta_2$, $\alpha_x\beta_2$
ICAM-1	$\alpha_L\beta_2$, $\alpha_M\beta_2$
ICAM-2,3,4,5	$\alpha_L\beta_2$
Invasin	$\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$
Laminin	$\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_6\beta_4$, $\alpha_v\beta_3$
MAdCAM-1	$\alpha_4\beta_7$
Matrix metalloproteinase-2	$\alpha_v\beta_3$
Neutrophil inhibitory factor	$\alpha_M\beta_2$
Osteopontin	$\alpha_v\beta_3$
Plasminogen	$\alpha_{IIb}\beta_3$
Prothrombin	$\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
Sperm fertilin	$\alpha_6\beta_1$
Thrombospondin	$\alpha_3\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
VCAM-1	$\alpha_4\beta_1$, $\alpha_4\beta_7$
Vitronectin	$\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_{IIb}\beta_3$
von Willebrand factor	$\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$

Table 2 : Association between integrins and their ligands; *Jay S. Desgrosellier and David A. Cheresh 2010*

induced by either ligand-mediated integrin clustering on the cell surface or increased intracellular signaling through molecules, such as the GTPase RAP1A (Han, Lim et al. 2006). Therefore, signaling that is induced by oncogenes or growth factor receptors may influence integrin affinity and function.

Integrin expression in cancer

A wide variety of integrins contribute to tumor progression. Many solid tumors originate from epithelial cells and the integrins expressed by epithelial cells as α6β4, α6β1, αvβ5, α2β1 and α3β1, are generally retained in the tumor and the expression levels may be altered. These integrins are typically involved in epithelial cell adhesion to the basement membrane, but might contribute to migration, proliferation and survival in tumor cells.
However, integrin expression can also vary very much between normal and tumor tissue. Most notably, integrins αvβ3, α5β1 and αvβ6, are usually expressed at low or undetectable levels in most adult epithelia but can be highly upregulated in some tumors. Expression levels of some integrins, such as α2β1, decrease in tumor cells (Kren, Baeriswyl et al. 2007). In fact, re-expression of α2β1 in breast cancer cells reversed some of the malignant properties of those cells, suggesting that α2β1 could function as a tumor suppressor (Zutter, Santoro et al. 1995). Studies correlating integrin expression levels in human tumors with pathological outcomes, such as patient survival and metastasis, have identified several integrins that might have an important role in cancer progression. Tumor cell expression of the integrins αvβ3, αvβ5, α5β1, α6β4, α4β1 and αvβ6 is correlated with disease progression in various tumor types (Table 3), therefore, these are the most studied integrins in cancer.

Integrin regulation of cell survival and apoptosis

Integrins constantly cooperate with their environment through their capacity to interact with the ECM activating cell survival or apoptosis signals. The balance of these signals maintain the integrity of different organs and tissues by preventing cells from surviving in an improper environment. Integrin ligation enhances cell survival through several mechanisms, including increased expression of BCl-2 (REFs 13,14) (Matter and Ruoslahti 2001) (Uhm, Dooley et al. 1999) or FlIP (also known as CFlAR) (Aoudjit and Vuori 2001), activation of the PI3K–AKT pathway or nuclear factor-κB (nF-κB) signaling (Courter, Lomas et al. 2005) and/or p53 inactivation (Bao and Stromblad 2004). These cell survival pathways are differentially regulated by specific integrin and growth factor receptor. It was shown that in

Tumour type	Integrins expressed	Associated phenotypes
Melanoma	αvβ3 and α5β1	Vertical growth phase [35,172–174] and lymph node metastasis[173,175]
Breast	α6β4 and αvβ3	Increased tumour size and grade[176], and decreased survival[177] (α6β4). Increased bone metastasis[36–38,64] (αvβ3)
Prostate	αvβ3	Increased bone metastasis[39]
Pancreatic	αvβ3	Lymph node metastasis[40]
Ovarian	α4β1 and αvβ3	Increased peritoneal metastasis[178] (α4β1) and tumour proliferation[179] (αvβ3)
Cervical	αvβ3 and αvβ6	Decreased patient survival[41,180]
Glioblastoma	αvβ3 and αvβ5	Both are expressed at the tumour–normal tissue margin and have a possible role in invasion[181]
Non-small-cell lung carcinoma	α5β1	Decreased survival in patients with lymph node-negative tumours[182]
Colon	αvβ6	Reduced patient survival[109]

Table 3 : Expression of integrins in different tumour types and the different phenotypes associated ; *Joy S. Desgrosellier and David A. Cheresh*

endothelial cells integrin αvβ3 crosstalk with fibroblast growth factor receptor (FGFR) prevents apoptosis (Alavi, Hood et al. 2003).

Although integrin antagonists directed to αvβ3 and αvβ5 promoted endothelial cell death, which led to decreased angiogenesis, deletion of *Itgb3* gene (which encodes integrin β3) or deletion of both *Itgb3* and *Itgb5* in mice did not inhibit angiogenesis. However, mice deficient in these integrins showed increased VEGF-mediated angiogenesis (Reynolds, Wyder et al. 2002), reflecting a compensatory increase in VEGFR2 in these mice (Reynolds, Reynolds et al. 2004). *Itgb3*–/– mice did show abnormal cardiac endothelial cell morphology associated with increased VEGF signaling (Weis, Lindquist et al. 2007). These results indicate a fundamental difference between studies involving genetic deletion of an integrin during early development and studies in which integrin antagonists were used to suppress integrin function in adult animals. It was illustrated the important role that the compensation could have in the interpretation of such knockout studies in mice.

The dual role of integrins in modulating apoptosis or cell survival was proved in some study were αvβ3 and α6β4 enhance tumor progression (Petitclerc, Stromblad et al. 1999), paradoxically, others such as α5β1 inhibit oncogene-induced transformation (Varner, Emerson et al. 1995). Further experiments showed that the pro-tumorigenic integrin αvβ3 could inhibit tumor progression in some mouse models of glioblastoma (Kanamori, Vanden Berg et al. 2004) and melanoma (Danen, van Kraats et al. 1996). This dual effect could be explained by the unligated integrins that can induce apoptosis (Stupack, Puente et al. 2001) (Zhao, Ross et al. 2005). In a process termed integrin-mediated death (IMD), unligated integrins on adherent cells recruit and activate caspase 8, resulting in apoptotic cell death (Stupack, Puente et al. 2001). IMD differs from anoikis, which is apoptosis that occurs in response to cellular detachment from the ECM (Frisch and Screaton 2001). Further studies demonstrated that the loss of caspase 8 is one mechanism by which tumor cells can avoid IMD, allowing increased metastatic dissemination (Stupack, Teitz et al. 2006). It is still unclear what part IMD plays in the therapeutic effects of integrin antagonists. However, it is thought that by inhibiting adhesion to the ECM, integrin antagonists can induce IMD and therefore have a greater effect in IMD-sensitive tumors. It was shown that in IMD-resistant tumor cells the unligated integrin αvβ3 substantially increases anchorage-independent tumor cell survival *in vitro* and metastasis *in vivo* (Desgrosellier, Barnes et al. 2009). These effects specifically required integrin αvβ3 recruitment and the activation of the non-receptor tyrosine kinase sRC, which leads to a FAK-independent survival pathway.

This anchorage-independent integrin αvβ3–sRC signaling might explain the association between integrin αvβ3 and tumor progression, as observed in various clinical studies (Takayama, Ishii et al. 2005) (McCabe, De et al. 2007).
αvβ3 antagonists that function by blocking ligand binding to tumor cells might be ineffective in treating some αvβ3-positive tumors. It remains possible that such antagonists could still function as anti-angiogenic agents. Integrin αvβ3-expressing tumors that recruit and activate sRC in this manner may be particularly sensitive to inhibitors such as dasatinib.

Integrin regulation of cancer stem cells

Many works indicate the integrins as markers of both normal progenitor and stem cell populations and cancer stem cells. The integrin αvβ3 represents a marker of luminal progenitor cells in the mammary ductal epithelium. Integrin signaling seems to maintain the cancer stem cell population in tumors, as ablation of FAK gene decreasing the pool of cancer stem cells in spontaneously forming mouse mammary tumors (Luo, Fan et al. 2009). Additionally, integrins may regulate the expression of cancer stem cell markers, such as CD44 (Samanna, Wei et al. 2006). The expression of specific integrins could enhance cancer stem cell properties through cooperation with tumor-initiating oncogenes or growth factor receptors.

The host cellular response to cancer

Integrins are important in different cellular functions, including angiogenesis, desmoplasia and the immune response in endothelial cells, fibroblasts, pericytes, bone marrow derived cells, inflammatory and platelets cells.

Angiogenesis

Tumor-associated blood vessels are structurally and biologically distinct from quiescent vessels, and according to Harold Dvorak “tumors make bad blood vessels” (Ribatti 2007). It has been reported that tumor associated vessels express integrin αvβ3 and αvβ5 that allow angiogenic endothelial cells to bind matrix proteins such as vitronectin, fibrinogen, von willebrand factor, osteopontin and fibronectin that are deposited in the tumor microenvironment. Through genetic deletion, or treatment with integrin antagonists, several additional integrins have been identified as crucial for angiogenesis, including α1β1,

α2β1,α4β1, α5β1, α6β1, α9β1 and α6β4 (Avraamides, Garmy-Susini et al. 2008). Integrin cooperation with particular growth factor receptors seems to confer responsiveness to specific angiogenic growth factors that are highly expressed in tumors. For example, αvβ3 and FGFR interaction induces angiogenesis downstream of FGF binding, and αvβ5 and VEGFR2 promote VEGF-induced angiogenesis (Friedlander, Brooks et al. 1995). The development of cilengitide as an anti-tumor and antiangiogenic agent directed to both integrins αvβ3 and αvβ5 is supported by on these findings. These distinct pathways of angiogenesis highlight the fact that integrins can integrate the ECM and growth factors to drive specific intracellular signalling events.

Perivascular cells

Angiogenesis depends on the invading endothelium but requires perivascular cells, as pericytes and vascular smooth muscle cells, that help the developing endothelium and promote blood vessel maturation. Tumors express immature blood vessels with reduced perivascular coverage (Baluk, Hashizume et al. 2005) leading to a poor perfusion and to the hypoxia typically observed in tumors. Integrins regulate the interaction between endothelial cells and the vascular basement membrane, and recent studies suggest that the endothelial cell integrin α4β1 is necessary for an interaction with vascular cell adhesion molecule 1 (vCAM1) on pericytes, resulting in endothelial cell–pericyte interaction and vessel stabilization (Garmy-Susini, Jin et al. 2005). Recent studies described an important role for blood vessel recruitment of pericytes in regulating blood vessel branching improving the delivery of chemotherapeutics. It has been shown that normalizing the tumor vasculature with agents such as bevacizumab or other VEGF pathway inhibitors should make it possible to increase drug delivery to the tumor and gain an improved therapeutic index for a wide range of anti-tumor agents.

Desmoplasia

A hallmark of the desmoplastic reaction in primary tumors and their metastases is the abundant collagen deposition. Through the deposited collagen activates integrin signalling increasing tumor cell proliferation, survival and chemoresistance (Conti, Kendall et al. 2008). Integrin α11 is commonly overexpressed in stromal fibroblasts that are associated with non-small-cell lung carcinoma (nsClC). Expression of α11β1 on fibroblasts increased tumor growth by stimulating the release of insulin-like growth factor 2 (IGF2) (Zhu, Popova et al. 2007).

This study highlights the importance of the regulation of growth factor signaling by integrins for the tumor-promoting effects of the host stroma. Targeting the tumor stroma with integrin antagonists could represent a new avenue for tumor therapy.

Bone marrow-derived and platelets cells

Immune cells, including macrophages and natural killer cells, can be involved in tumor suppression. The expression of integrin αvβ3 on macrophages is important for their tumor suppressive function. In fact was demonstrated that macrophage tumor infiltration is decreased in *Itgb3*-/- mice (Taverna, Moher et al. 2004).
Multiple studies associate tumor cell–platelet interactions with an increases of tumor metastasis. The ECM protein fibrinogen functions as a bridge between integrins αIIbβ3 on platelets and αvβ3 on tumor cells. This interaction facilitates tumor cell arrest in the vasculature, leading to metastasis to various sites, including the bone marrow (Bakewell, Nestor et al. 2003). Combined inhibition of the tumor integrin αvβ3, expressed from the tumor, and the integrin αIIbβ3, exposed on platelet, increased the antiangiogenic and anti-tumor effects compared with blocking tumor integrin αvβ3 alone (Trikha, Zhou et al. 2002), suggesting that antagonists that target both integrins on platelets and endothelial cells could have greater clinical efficacy in neoplasia treatment.

Integrin crosstalk with growth factor cytokines

Integrins cooperate with growth factor receptors and cytokine receptors in many aspects of tumor progression. In tumor cells integrin regulates adhesion, migration, invasion and survival, but also can affects the host response vs cancer, in particular in the angiogenic endothelium. Integrins form a complex with growth factor receptor (Borges, Jan et al. 2000) (Trusolino, Bertotti et al. 2001) potentiating activation of downstream kinases such as MAPK (Miyamoto, Teramoto et al. 1996) or AKT (Caswell, Chan et al. 2008) and therefore enhancing cell migration and survival.

EGF and its receptors

Cooperation between integrins and EGF receptor family members affect many aspects of tumor progression, including tumor initiation, proliferation, migration and invasion. It was reported that integrin α6β4 cooperates with ERBB2 in mammary tumor formation and tumor cell invasion of breast cancer patients. This cooperative effect could be due to the formation

of an integrin α6β4–ERBB2 complex that enhances the pathway activation with the loss of cell polarity and hyper-proliferation. Inhibition of this complex by *Itgb4* deletion increases the efficacy of ERBB2 targeted therapy. This results underline the value of combinatorial therapy using integrin and EGF receptor family members antagonists (Guo, Pylayeva et al. 2006). Also in pancreatic cancer, the EGF pathway is often hyperactivated, and EGF together with integrin αvβ5 stimulates pancreatic tumor cell migration on vitronectin *in vitro* and metastasis *in vivo* (Ricono, Huang et al. 2009). In non stimulated cells integrin αvβ5 is unable to form cluster and focal adhesions on its own (Wayner, Orlando et al. 1991), and requires EGF-dependent activation of sRC for its ability to mediate cell migration (Ricono, Huang et al. 2009).

Not only in pancreatic cancer EGF–integrin crosstalk is active, it also increases colon cancer migration cells through integrins α3β1 and α6β4 (Pouliot, Nice et al. 2001), and hepatocellular carcinoma through integrins α1β1 and α2β1 (Yang, Zeisberg et al. 2003).Therefore, EGF signaling may increase tumor cells integrins mediated cell migration and survival, resulting in increased metastatic potential. Other studies have demonstrated that integrin stimulation itself regulates EGF signaling, in fact, integrin ligation can induce EGFR phosphorylation independently of EGF, resulting in increased MAPK activation, tumor cell proliferation and survival98 through a sRC–p130CAs pathway (Moro, Dolce et al. 2002). A recent study showed that integrin signaling increased EGF secretion and ERBB2 clustering in breast cancer cells, resulting in resistance to the ERBB2 inhibitor, trastuzumab (Wang, Xiang et al. 2009). Inhibition of integrin signaling reversed trastuzumab resistance, suggesting that this combined approach may prove therapeutically efficacious in ERBB2-expressing breast cancers.

HGF and receptors

Integrins cooperate with MET, the hepatocyte growth factor (HGF) receptor resulting in enhanced tumor progression. In particular, integrin β4 synergizes with MET to enhance the transformation of fibroblasts and increase tumorigenic potential. For example in breast cancer cells, HGF binding to MET increases anchorage-independent growth by inducing phosphorylation of integrin β4, resulting in the recruitment of proteins such as sRC and ERK (Bertotti, Comoglio et al. 2006). Complex between MET and integrin α6β4 enhances HGF-induced signals, including tumor cell invasion. This is due to the potentiation of HGF-induced Ras and PI3K signalling by α6β4-mediated recruitment of proteins as PI3K

(Trusolino, Bertotti et al. 2001). MET cooperates with other integrins as integrin αvβ5 contributes to MET signaling by controlling the expression of HGF-induced genes required for cell migration (Crouch, Spidel et al. 2004).

TGFβ and receptors

Transforming growth factor-β (TGFβ) is a well-characterized inducer of epithelial–mesenchymal transformation (EMT) in tumor cells, resulting in enhanced cell migration and invasion. Integrins are involved in the TGFβ activation. TGFβ ligands are secreted as inactive complexes with a latency-associated peptide (lAP). The TGFβ1 lAP was first identified as a ligand for integrin αvβ6, and expression of integrin αvβ6 regulates TGFβ1 activation (Munger, Huang et al. 1999). Increased integrin αvβ6 expression, in basal cell carcinoma, correlates with aggressive disease, possibly due to increased TGFβ1 activation forming a dense tumor stroma (Marsh, Dickinson et al. 2008). Integrin αvβ3 and sRC cooperate with TGFβ to induce EMT of mammary epithelial cells (Galliher and Schiemann 2006), and this requires sRC-dependent phosphorylation of TGFβ receptor type 2 (TGFBR2) (Galliher and Schiemann 2007). Additionally, TGFβ stimulation induces phosphorylation of the cytoplasmic domain of integrin β1, resulting in integrin activation and tumor cell invasion. TGFβ is predominantly secreted by tumor stromal cells and the crosstalk between integrins and TGFβ may have an important role in the contribution of the tumor stroma to cancer progression.

VEGF, FGF and their receptors

Specific integrins mediate distinct pathways of tumor angiogenesis. During tumor angiogenesis endothelial cells in the tumor microenvironment must resist to cell death. This can be induced by stresses such as hypoxia and nutrient deprivation (intrinsic apoptosis) or inflammatory mediators (extrinsic apoptosis). These endothelial cells are protected from distinct apoptotic stimuli through differential activation of Raf (Hood, Frausto et al. 2003). FGFR cooperates with integrin αvβ3 to increase the phosphorylation of Raf ser338 and ser339, resulting in Raf complex formation in the mitochondria, inhibiting the intrinsic pathway of apoptosis. By contrast, vEGFR2 cooperates with integrin αvβ5, leading to sRC-dependent phosphorylation of Raf Tyr340 and Tyr341 and resistance to extrinsic apoptosis that is induced by inflammatory mediators such as tumor necrosis factor (TNF) (Bao and Stromblad 2004). The activation of integrin αvβ3 can in turn increase tumor cell secretion of

vEGF, providing a feedback loop resulting in increased tumor growth (De, Razorenova et al. 2005).

CXCR4

CXCR4 is known for its role in the recruitment of haematopoietic cells to infected sites but is also expressed on tumor cells and various tumor associated cell types. The binding of its ligand stromal cell-derived factor 1 (sDF1; also known as CXCl12) induces tumor cell migration and contributes to metastasis. sDF1 stimulation increases the expression of integrins, such as α5β1 and αvβ3, increasing cell adhesion and invasion *in vitro* (Engl, Relja et al. 2006) (Sun, Fang et al. 2007) and metastasis *in vivo* (Cardones, Murakami et al. 2003).

AIM OF THE WORK

Our aim was to produce antibodies that specifically targeted markers on Glioblastoma Cancer Stem Cells. These cells behave differently from the differentiated counterpart and from the ATCC lines in terms of resistance to the drugs and for their altereted pathways. For that reason they are a potent tool to generate specific antibodies.
The new marker that we found to be expressed on BTSC is integrin alpha 7. The mAb that we generated against integrin alpha 7 could help to set up new treatment in combination with the standard chemo/radiotherapy adding efficacy to present therapies. Moreover the discovery of a common stemness marker as antigen on glioblastoma stem cells allow to select for cancer stem cells and this help to isolate and characterize them.

Materials and Methods

Cells Culture

BTSC clones were cultured in serum-free medium containing 20µl/ml glucose 30%, 15µl/ml sodium bicarbonate 7.5%, 5µl/ml Hepes 1M, 2µg/ml heparin, 4mg/ml BSA, 10µl/ml glutamine and P/S dissolved in DMEM–F12 medium and supplemented with 20 ng/ml EGF and 10 ng/ml bFGF and 100 µl/ml Hormone mix 10X containing 200 µl/ml DMEM F12 5X, 20 µl/ml glucose 30%, 15 µl/ml sodium bicarbonate 7.5%, 5µl/ml Hepes 1M, 1mg/ml apotrasferrin, 50mg/l insulin, 96,6mg/l putrescine, 100µl/l selenium 3x10-3M, 100µl/l progesterone 2x10-3M. Cultures were expanded by mechanical dissociation of spheres followed by re-plating of both single cells and residual small aggregates in complete fresh medium and were incubated at 37°C with 5% CO_2. HEK293T human renal epithelial cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS). U251, L929 and U87MG cells were maintained in DMEM +10% FBS; LN215 and T98G cells in RPMI 1640 + 10% FBS. SHSY-S5Y, SHEP and Kelly were kind gifts from Prof. Dr. Simone Fulda and maintained in RPMI 1640 + 10% FBS. The myeloma cell line X63-Ag8.653 was a kind gift from Dr. Martin Sprick and maintained in RPMI +10% FBS. The established hybridoma lines were cultivated under identical conditions. All cells were incubated at 37°C, 5% CO_2.

Lentiviral vector production and Infection

HEK293T cells were cotransfected with 28 µg of the packaging vector (psPAX2), 14 µg of the envelope vector (pMD2) and 40 µg of the DNA of interest using the standard $CaPO_4$ transfection methods. Virus-containing supernatant was collected after 72 h, centrifuged at 1800 rpm for 5 minutes at 25°C and filtered (0,45 µm). The cells incubated with virus were centrifuged at 1800 rpm for 30 minutes in presence of 10 µg/µl polybrene (SIGMA) and put in the incubator over night. The media was changed 24h later and after 48h we started the selection of the cells with 10 µg/µl puromycin. The lentiviral vector contains the shRNA B10 (sh#1) or C1 (sh#2) to knockdown ITGA7 were obtained from Thermo Scientific. All experiments were performed 10-20 days post transfection.

Immunoprecipitation

For the preparative immunoprecipitation experiments, $5x10^7$ BTSC1 cells were lysed in 1 ml IP-lysis buffer (30 mM Tris-HCl [pH 7.4], 120 mM NaCl, 2 mM EDTA, 2 mM KCl, 1% Triton X-100, 1× COMPLETE protease-inhibitor cocktail [Roche]) at 4°C for 30 min. The lysates were centrifuged at 15,000 × g for 30 min. Preclear was performed two times with magnetic protein G beads (Invitrogen; Carlsbad, CA) in presence of 5 µg isotype control antibody (IgG2a) and one time with magnetic protein G beads alone for 30 min/each. ITGA7 was precipitated using 1.4A12 antibody for 4 hrs at 4°C. The beads were washed five times with 1 ml IP-lysis buffer and eluted with 2× LDS buffer (NuPAGE, Invitrogen; Carlsbad, CA). Proteins were separated by SDS-PAGE (NuPAGE) and analyzed by silver staining and coomassie staining. For the analytical immunoprecipitation, $2x10^6$ BTSC1 cells were surface biotinylated with Sulfo-NHS-LC-Biotin (Pierce/Thermo Fisher; Waltham, MA) according to the manufacturer´s recommendation. Immunoprecipitation was performed as described above and the biotinylated proteins were detected by western blot using streptavidin-HRP (GE Healthcare). For ITGB1 immunoprecipitation the MEM101A antibody (santa cruz biotechnology sc-51649) was used.

Western Blot analysis

Protein lysates were prepared using standard RIPA buffer (150 mM NaCl; 20 mM Tris, pH 7.2; 0.05% SDS; 1.0% Triton X-100; 1% Deoxycholate; 5 mM EDTA) and the proteins were separated with NuPAGE gels, using MOPS buffer (Invitrogen). Proteins were blotted according to the manufacturers' recommendation (Invitrogen). The membranes were blocked for 1 hour with PBST (PBS, 0.02% TWEEN20) containing 5% blotting grade nonfat powdered milk. Antibodies used were anti Primary antibodies used were obtained from Santa Cruz Biotechnology: pRb Ser 807/811 (# 9308), PLK1 rabbit (#4513), FAK (#3285), pFAK (#8556), Src (#2123), pSrc (#6943), SOX2 (#3579); Santa Cruz Biotechnology: cyclin B1(sc-166757) and SIGMA: ITGA7 (#HPA008427); □-tubulin (#T4026).We used for the detection Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate, an enhanced chemiluminescent (ECL) substrate for horseradish peroxidase (HRP) enzyme that provides low picogram detection of proteins and Thermo Scientific SuperSignal West Dura Substrate, a luminol-based enhanced chemiluminescence (ECL) HRP substrate optimized for high sensitivity. The secondary antibody used were obtained from Southern Biotechnologies) and 1:10.000 diluted.

Immunofluorescence

For immunostaining analysis the spheres cells where seeded on poly-lysine treated glasses for 10 minutes to allow the attachment. The differentiated cells were grown on the glasses in absence of EGF and bFGF in DMEM F12 containing 10% fetal calf serum (Sigma) for 14 days at 37°C . Cells were washed 3 times with PBS and then fixed with 4% PFA for 15 minutes at room temperature, washed 3 times with PBS, permeabilized with PBS 0.1% triton x-100 (Sigma) for 15 minutes at room temperature in the dark. Cells were blocked with a PBS solution containing 3% BSA (Sigma) plus 0.05% tween for 1 hour at room temperature. Cells were incubated overnight at 4°C with the appropriate antibody: mouse monoclonal anti Integrin α7 1.4 purified (or SN), rabbit polyclonal anti-Nestin (Sigma N5413), mouse monoclonal anti- GFAP (BD Pharmigen 560298, clone 1B4), rabbit monoclonal anti-SOX-2 (Cell Signaling D6D9), mouse monoclonal anti-Musashi (RD System MAB2628, clone282613), polyclonal rabbit anti-Laminin (Sigma L9393). Cells were washed 3 times with PBS and incubated with the correct secondary antibody (1:100) for three hours at 4°C protected from the light: goat anti-mouse IgG2bAlexa 647 (Life technologies A21242), goat anti-rabbit Alexa 488 (Invitrogen A-11008); Nuclei were stained with DAPI (Life technologies D1306) at final concentration of 10µg/ml. Cells were washed 3 times with PBS and mounted with ProLong AntiFade (Invitrogen P36934). Imaging was done using a FV1000 Leica confocal microscope.

Spread assay

The silenced cells and the wild type control cells were incubate on matrigel coated plate with a fresh matrigel on the top of the cells over night at 37°C. After the images were taken with fluorescence microscope.

Invasion assay

For the invasion assay the upper wells of the plate (Invitrogen) were coated with 20µg/ml of laminin (Sigma) diluted in PBS for 3 hours at RT. After the wells were washed 3 times with PBS to remove the excess of the laminin. The lower wells were filled with cancer stem cells media plus 3x (60ng/ml EGF and 30ng/ml FGF) growth factors and for the antibodies treatment with control or 1.4A12 or Fab abs at final concentration of 50 µg/ml. $4x10^4$cells were counted, washed 3 times with PBS and resuspended with cancer stem cells media and for antibodies treatment with control or 1.4 or Fab antibodies at final concentration of 50µg/ml. The cells were incubated at 37°C for 48 hours. The cells that underwent to the

invasion were stained with 40 µM calcein (Invitrogen) and analyzed with the fluorescence microscope. For the ITGA7 knock down cells and ctrl wild type the upper wells of the plate (Invitrogen) were coated with 20µg/ml of laminin (Sigma) diluted in PBS for 3 hours at RT. After the wells were washed 3 times with PBS to remove the excess of the laminin. The lower wells were filled with cancer stem cells media plus 3x (60ng/ml EGF and 30ng/ml FGF) growth factors and $4x10^4$cells of were counted, ITGA7 knockdown cells and ctrl wild type, washed 3 times with PBS and resuspended with cancer stem cells media in the upper part of the wells.

Differentiation of primary BTSC lines

BTSCs were resuspended in DMEM F12 + 10% FBS and plated onto a basement membrane-like extracellular matrix extract (matrigel)-coated 6 well plate (50.000 cells/well) and incubated for 10-14 days at 37°C, 5% CO_2.

Cell proliferation assay

After plating equal number of cells, the Cell Titer-Glo® Luminescent Cell Viability Assay (Promega) was performed. Control wells containing medium without cells were prepared to obtain a value for background luminescence. We normalized the values obtained for each time point to day 0. The results are the means and SEM of 3-5 independent experiments.

Flowcytometric analysis

Cells were mechanically disgregated, washed and resuspended in PBS/BSA 0,5% buffer. For extracellular stainings, cells were incubated for 45 min with primary antibody in PBS BSA 0.5% or the hybridoma SN. Cells were washed twice in PBS/BSA 0.5% and incubated with the secondary antibody in PBS BSA 0.5% for 30 min. For the intracellular staining the cells were fixed in 200 µl PBS containing 2% PFA for 10 minutes and permeabilized with PBS/Triton 0.1%. Primary antibody was diluted in PBS/Triton 0.1% and the cells were stained 45 minutes on ice. After two washes in PBS Triton 0.1% at 4° C, we diluted the secondary antibody in PBS/Triton 0.1% and the cells were stained 30 minutes on ice. The cells were washed two times and resuspended in 100 µl PBS BSA 0.5% and analyzed by flow cytometry. Viable cells were detected by 7-Amino-actinomycin D (7-AAD, 5 mg/ml) exclusion. The results were analyzed with FloJo software. Antibodies used were: mouse anti GFAP cocktail (BD Biosciences #556330), ITGB1 (Santa Cruz Biotechnology sc-51649)

PE-conjugated anti CD133/AC133 (Miltenyi # 130-098-826), NL637 conjugated anti Nestin (R&D Systems #NL1259V), anti ITGA7 (1.4A12), P5B6 SN, P9E6 SN and P3H9 SN.

Cell cycle analysis

Cells were harvested and mechanically dissociated. Then the cells were lysed in 0.1M citrate buffer pH7.4 containing 0.1% Triton X100, 50 µg/ml propidium iodide (Sigma) and 50µg/ml RNaseA (Sigma) on ice for 30 min. Then samples were measured by flowcytometry.

Hybridoma generation

For the generation of the integrin alpha7 specific antibody 1.4A12, 6 Balb/C mice were immunized by intraperitoneal (i.p.) injections of $5x10^5$ living GSC1 cells. 40, 70 and 84 days after the initial immunization the antibody production was boosted by i.p.. injection of $1\text{-}2x10^5$ GSC1. The final boost was performed as i.v. injection using a 50 µg total protein from a membrane protein preparation of BTSC1 cells. Subsequently the mice were euthanized, the spleen was removed under aseptic conditions and the splenocytes were fused with X63-Ag8.653 myeloma cells using standard fusion methods. One day after fusion the cells were directly plated in 96 well plates to densities of 1-5 surviving hybridoma clones/well (3000 wells/complete spleen) and selected with HAT supplement (Invitrogen). After 10 days of HAT selection the hybridoma supernatants were tested for immunoreactivity towards primary brain tumour stem cells as described in "high throughput hybridoma screening". Positive hybridomas were 2 times subcloned and immunreactivity was confirmed by flowcytometric analysis of antibody surface binding on GSC1. The hybridomas were frozen and stocked in nitrogen. Antibody purification using a ReSURE protein A column (GE Healthcare) was performed according to the manufacturers' recommendations.

High throughput hybridoma screening

We labeled PBL and in vitro differentiated BTSC1 cells with 10 µg/ml cell tracker Oregon green A488 and HOECHST 33342, respectively for 20 min at 37°C. No-incorporated dye was removed by 3 washes with PBS. The cells were counted and mixed in a 1:1:1 ratio with a single cell suspension of non-labeled BTSC1 cells. The staining was performed in 96 well round bottom plates and 20000 cells/ well were incubated with 80 µl of the individual hybridoma supernatants for 1 hour on ice. The supernatants were removed and cells were

washed 2 times with PBS + 0.5% BSA. The bound antibody was then labeled with an PE-conjugated, mouse Ig-detecting secondary antibody (Invitrogen).The secondary antibody was 1:200 diluted in PBS+0.5% BSA and incubated for 30 min on ice. The cells were again washed and FACS analysis was performed using a FACS LSR II flowcytometer equipped with a HTS-96 plate holder. This methodology allowed for the analysis of more than 600 supernatants per day. The hybridomas producing antibodies, which bound preferentially to stem-like GSC1 but not or weakly to PBLCs were further analyzed for binding on other BTSC or differentiated lines.

In vivo study

For subcutaneous xenograft models, $1x10^6$ BTSC1 cells transduced were injected into the flanks of the mice (n = 12/group). Tumor growth was monitored and tumor size was measured with a caliper. Tumor growth was monitored after injection by in vivo bioluminescence method. In short, mice were injected with 150 mg/kg Luciferin intraperitoneal. After 15 min the bioluminescence was detected with a Xenogen IVIS 100 small animal in vivo imaging system.

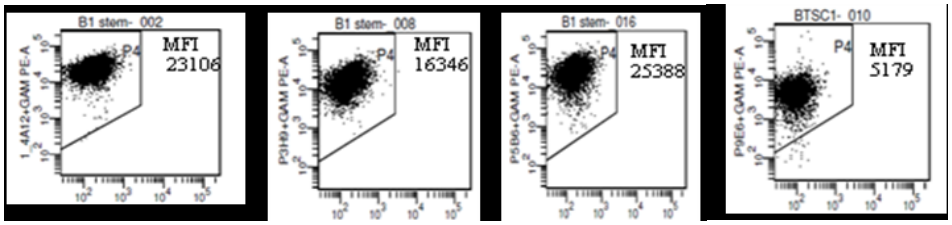
RESULTS

Generation and screening of a hybridoma library raised against BTSC surface antigens

We immunized Balb/c mice with 500.000 living BTSC1 cells by intraperitoneal injection (i.p.) in order to generate antibodies recognizing brain tumor stem cells. In an interval of 4 weeks, the mice were boosted two times with 100.000 BTSC1 cells i.p., before the last boost was given using isolated membrane fractions of the same cell line. Then the mice were sacrificed and the splenocytes fused with a myeloma cell line to generate hybridomas. The hybridomas obtained from the fusion of one spleen were directly cloned in 30 complete 96 well plates summing up in 3000 wells with growing hybridomas. Subsequently, the hybridoma supernatants were analyzed using an optimized high throughput FACS-based protocol. The protocol was developed in order to isolate antibodies recognizing surface molecules expressed on BTSC1and only weakly on the differentiated glioblastoma cell line U87MG, or peripheral blood lymphocytes. This gave us a fast and very stringent selection of less 2% of all initial wells seeded. In the next step the clones passing this first selection were subcloned and selected for immune reactivity to three more independent BTSC clones combined with weak positivity to three more differentiated brain tumor cell lines T98G, SHSY5Y and SHEP lines.

1.4A12 selection by hybridoma screening strategy

The clones displaying a strong binding to stem-like BTSC1 were cloned and expanded. The supernatants from hybridomas were collected and tested on BTSC1 in order to verify if the clones selected after the initial screening were still producing antibody. Most of the hybridoma supernatants were found to be positive (Fig.1), confirming the high quality of the initial screening procedure. The supernatants were tested for binding on commercially available differentiated glioblastoma cell lines, such as LN215 and T98G and re-screened for reactivity towards BTSC1(Fig.2). We were interested to select hybridomas preferentially binding to cancer stem cells used for immunization and not to differentiated glioblastoma cell lines. For this reason we excluded these hybridomas from our further analysis and we concentrated on 1.4A12 antibody (Fig.2).



1.4A12 P3H9 P5B6 P9E6

Fig.1 Reactive supernatants against BTSC1. Mean Fluorescence Intensity (MFI) was determined for the whole cell population. The secondary antibody, specific for mouse immunoglobulins was labeled with PE, (R-Phycoerythrin).

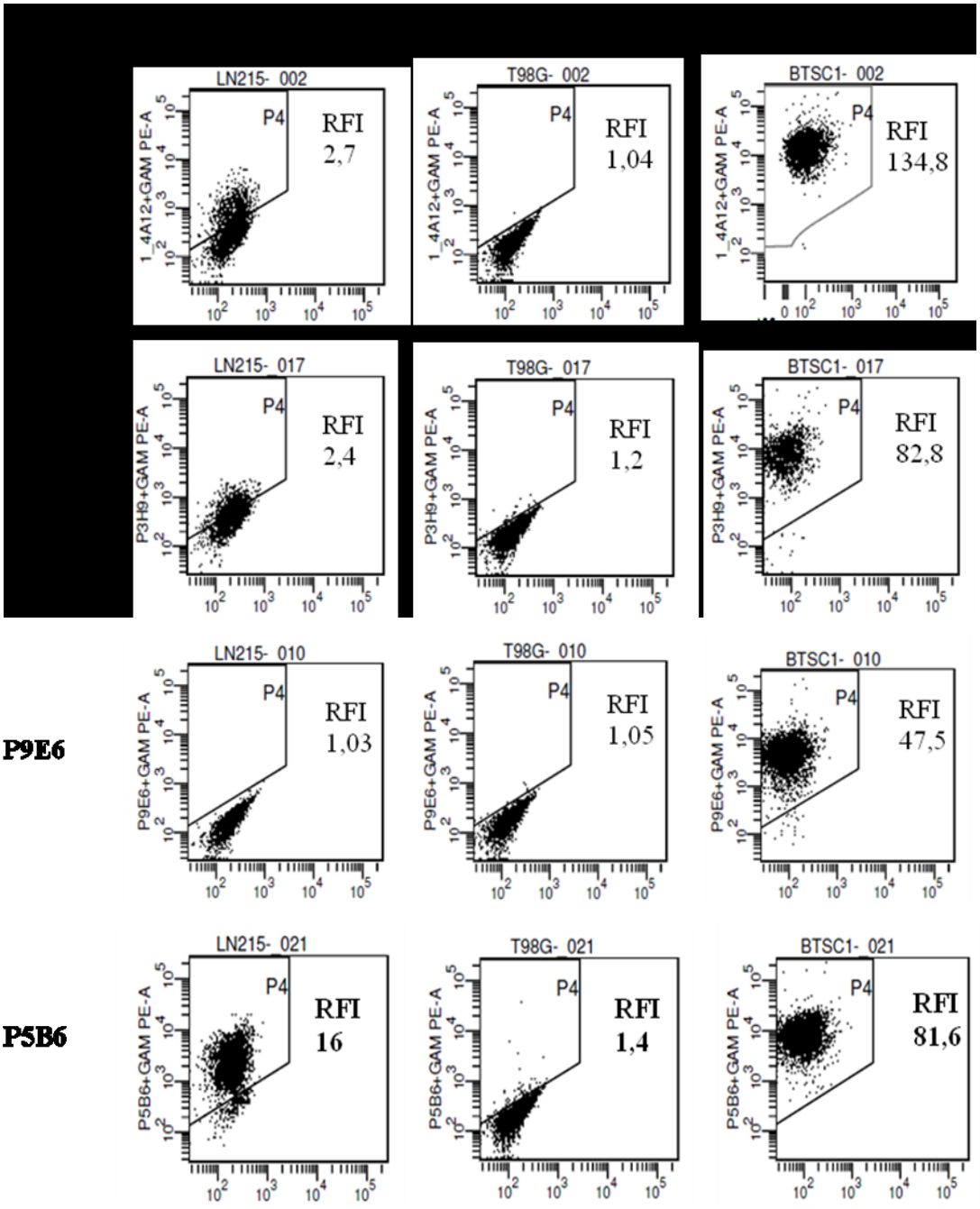


Fig.2 *S*creening for antibodies binding on differentiated glioblastoma cell lines and re-screening for reactivity towards BTSC1 to select the supernatants that better binds to the cancer stem cells. RFI represents the Relative Fluorescence Intensity obtained by the ratio of the Mean Intensity Fluorescence (MFI) obtained with the hybridoma supernatant and the MFI of the control

1.4A12 as surface marker of glioblastoma stem cells

One candidate clone (1.4A12) showed a very strong immune reactivity by flowcytometric analysis to all BTSC lines, while only weak positivity to the commercial cell lines tested (Fig 3). We evaluated the stemness/differentiation markers on our brain tumor stem cells (Fig 4a and b) and we analyzed the expression of 1.4 antibody among them (Fig 4c). We detected a preferential binding of the antibody on the surface of undifferentiated cells (Fig 4c and e) by immunofluorescence, by western blot (Fig 4d) and flowcytometric analysis (Fig 4e).

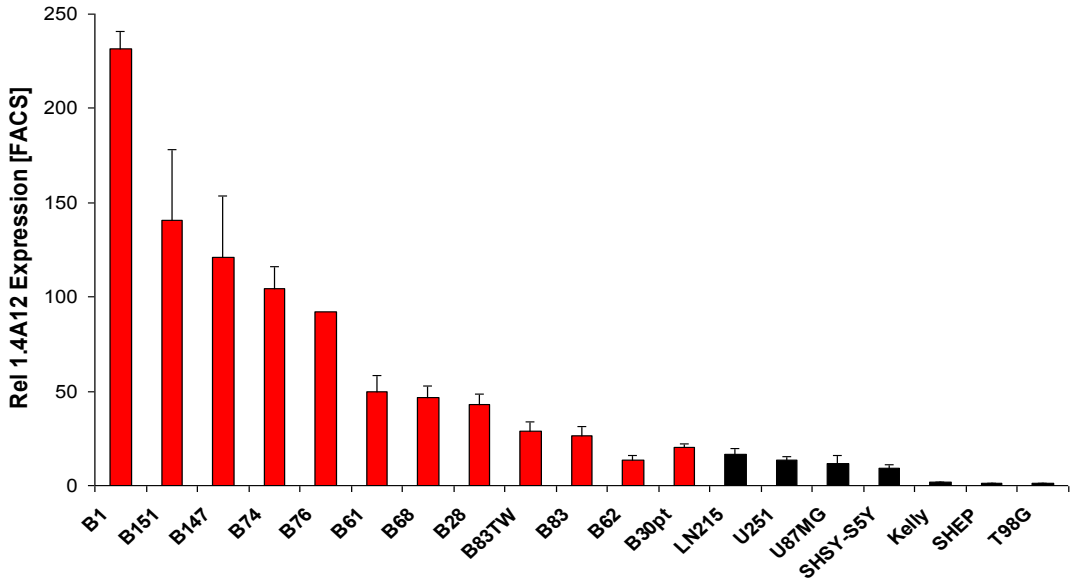
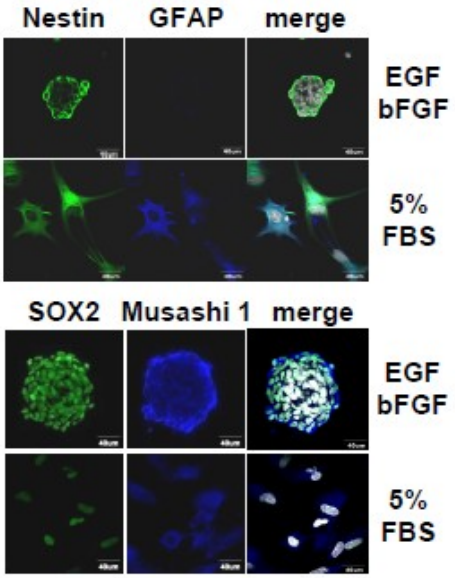


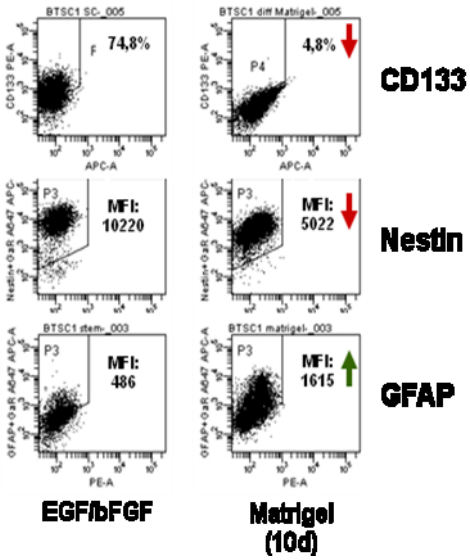
Figure 3: Binding pattern of 1.4A12 on primary BTSC and long term established brain tumor cell lines

Shown is the FACS analysis of the antibody produced by clone 1.4A12 on primary brain tumor stem cell lines and on differentiated commercial brain tumor cell lines.

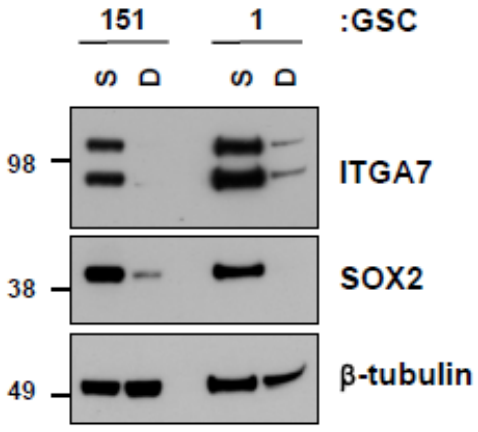
a)


Nestin
GFAP
merge
EGF
bFGF
5%
FBS
SOX2
Musashi 1
merge
EGF
bFGF
5%
FBS

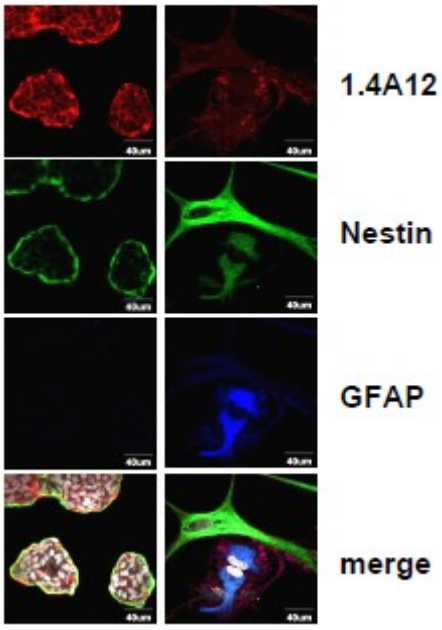
b)


74,8%
4,8%
CD133
MFI:
10220
MFI:
5022
Nestin
MFI:
486
MFI:
1615
GFAP
EGF/bFGF
Matrigel
(10d)

c)


151
1
:GSC
S
D
S
D
98
ITGA7
38
SOX2
49
β-tubulin

d)


1.4A12
Nestin
GFAP
merge

e)

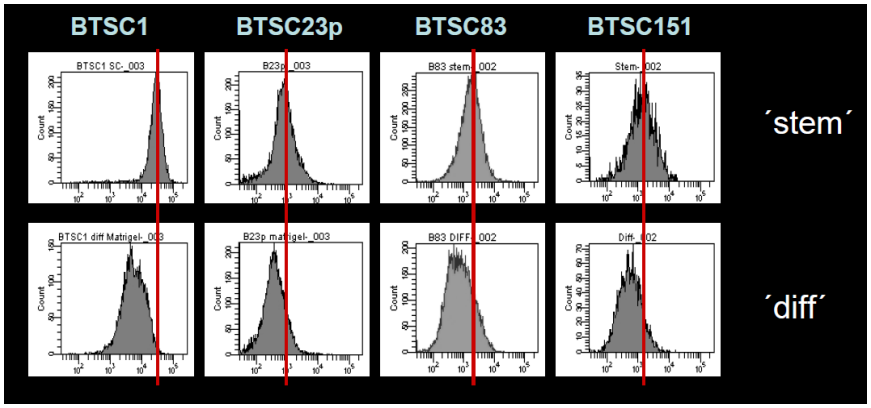


Figure 4: Immunoreactivity of 1.4A12 on differentiated BTSCs is reduced compared to their stem-like counterpart

a) Shown is the binding pattern of the antibodies on the primary brain tumor stem cell line BTSC1 grown in presence of EGF and FGF and in differentiated conditions by IF analysis using the antibodies indicated **b)** Same conditions as above performing a FACS analysis **c)** Binding pattern on BTSC clones under stem like and differentiation conditions using the 1.4 antibody. The binding was determined by IF and representative results are shown. The cells were differentiated in the presence of 5% serum for 10-12 days **d)** Western blot using 2 clones of BTSC in stem cells condition and differentiation media **e)** Binding pattern on different cancer stem cells and differentiated counterpart by Facs using 1.4A12 antidody

Identification of antigen recognized by 1.4A12 antibody

The antigen recognized by 1.4A12 antibody was identified by immunoprecipitation followed by mass spectrometric analysis. We determined the molecular weight of the antigen by performing a cell surface biotinylation prior to cell lysis and precipitation using the 1.4A12 antibody. By western blot analysis we were able to detect a strong signal at a molecular weight of approximately around 100-110 kDa (Fig 5a). We detected a protein signal in the silver gel and even in the coomassie stained preparative SDS-PAGE gel (Fig 5b, c). We excised the bands from the gel, digested the containing protein with trypsin and performed a tandem mass spectrometric analysis. We found a large amount of peptides (> 100) present in integrin alpha7, which has a theoretical molecular weight fitting to our observations. We performed an immunoprecipitation using isotype control, 1.4A12 or integrin beta 1 antibody coupled to protein G beads to verify the binding between integrin alpha7 and integrin beta 1proteins. As control we used T98G cells overexpressing Integrin alpha7 (Fig 5d). Shown is a western blot analysis, probed with the antibodies indicated. We also cloned the protein-encoding cDNA in a lentiviral expression vector (pTWEEN). Using this expression vector, we verified that the antigen recognized by1.4A12 was integrin alpha7 protein. Ectopic expression of the integrin alpha7 protein in 293T cells, which do not express it on their surface, determinate the binding of 1.4A12 antibody, which correlated with the positivity for the co-expressed GFP (figure 6a). We also downmodulated the expression of integrin alpha 7 in BTSC1 by stable transduction with lentiviral shRNA constructs. In contrast with the cells treated with control shRNA, which are strongly positive for 1.4A12 binding, the binding of the antibody is reduced in cells treated with integrin alpha 7shRNA (Fig 6b). Taken together, these results strongly indicated that 1.4A12 antibody recognizes integrin alpha 7 on the cell surface of all BTSCs tested.

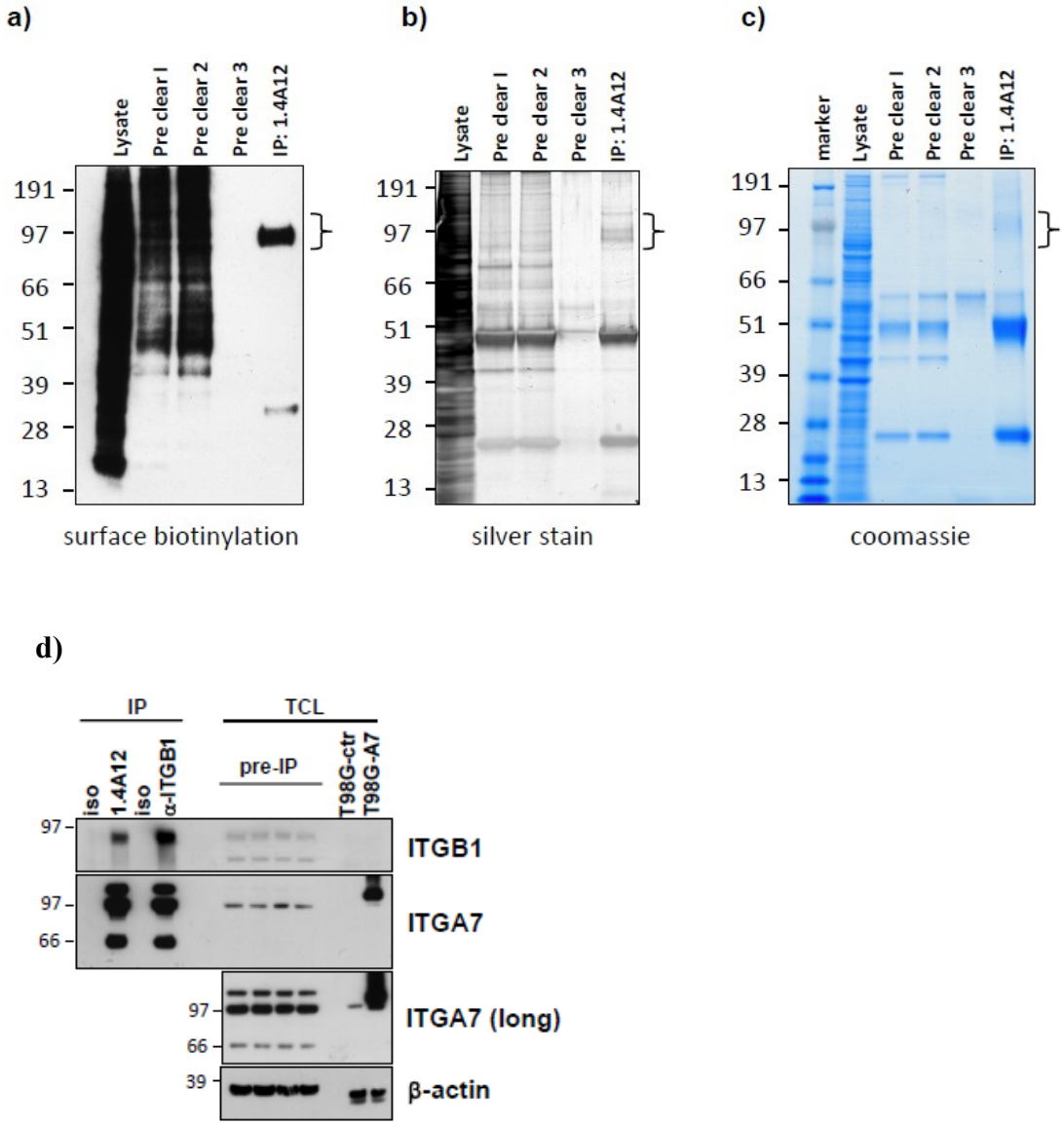


Figure 5: Biochemical isolation of the 1.4A12 antigen

a) $2x10^6$ BTSC1 cells were first surface biotinylated using Sulfo-NHS-LC-Biotin (Pierce) and then subjected to immunoprecipitation using either IgG2a control antibody (preclears) or the 1.4A12 antibody coupled to protein G beads. Shown is a western blot analysis, probed with strepatvidin-HRP in order to detect the biotinylated antigen. **b)** The IP was repeated with $5x10^6$ BTSC1 cells and the isolated proteins were visualized by SDS PAGE and silver staining. **c)** Coomassie staining of the SDS-PAGE with the preparative immunoprecipitation, used for mass spectrometric analysis. The brackets show the bands with the potential 1.4A12 antigen(s). **d)** Immunoprecipitation using isotype control, 1.4A12 or integrin beta 1 antibody coupled to protein G beads. Shown is a western blot analysis, probed with the antibodies indicated. As specificity control serve ITGA7 transduced T98G cells.

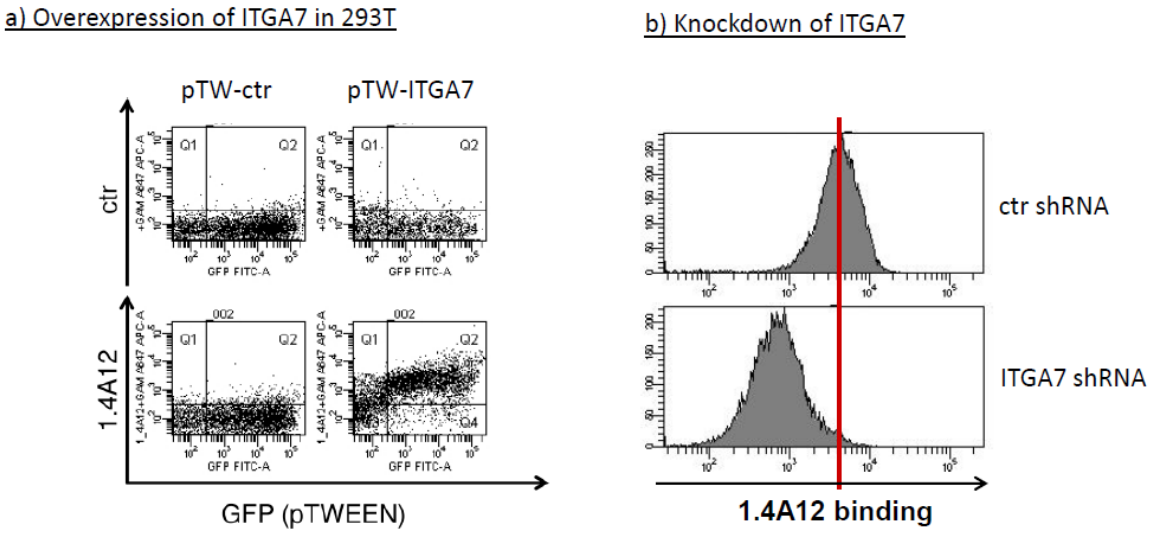


Figure 6: Genetic evidence for ITGA7 as the antigen of 1.4A12 antibody

a) HEK293T cells were transiently transfected with an expression plasmid for GFP (pTW-ctr) or ITGA7. 48 hrs post transfection the cells were harvested and a flowcytometric analysis using either a control (ctr) or the 1.4A12 antibody (1.4A12) was performed. **b)** BTSC1 cells which display a strong positivity for 1.4A12 binding were transduced with either control (ctr) or ITGA7 shRNA. 120 hours post transduction a flowcytometric analysis using 1.4A12 antibody (1.4A12) was performed. The results shown are representative for at least 3 biological replicates.

1.4A12 positive SH-SY5Y grow better in vitro and engraft faster in vivo

Neuroblastoma line SH-SY5Y contains a small subpopulation (10-15%) of cells strongly positive for integrin alpha 7 (Fig 7a). We sorted these cells by flowcytometry and found them growing significantly faster *in vitro*, when compared to the integrin alpha 7 negative sorted cells (Fig 7b). Interestingly, the cells positive sorted lost the expression of integrin alpha 7 over time, indicating, that only the fast proliferating subpopulation of SH-SY5Y are positive for this protein (Fig 7a). Importantly, we were also able, to verify the enhanced aggressiveness of 1.4A12 positive SH-SY5Y *in vivo*. Cells positive for integrin alpha 7 engrafted significantly faster in immune-deficient NSG mice, when compared to cells negative for this protein (Fig 7c). Thus, our data strongly suggests that integrin alpha 7 expressing cells represent an aggressive subpopulation of SH-SY5Y cells.

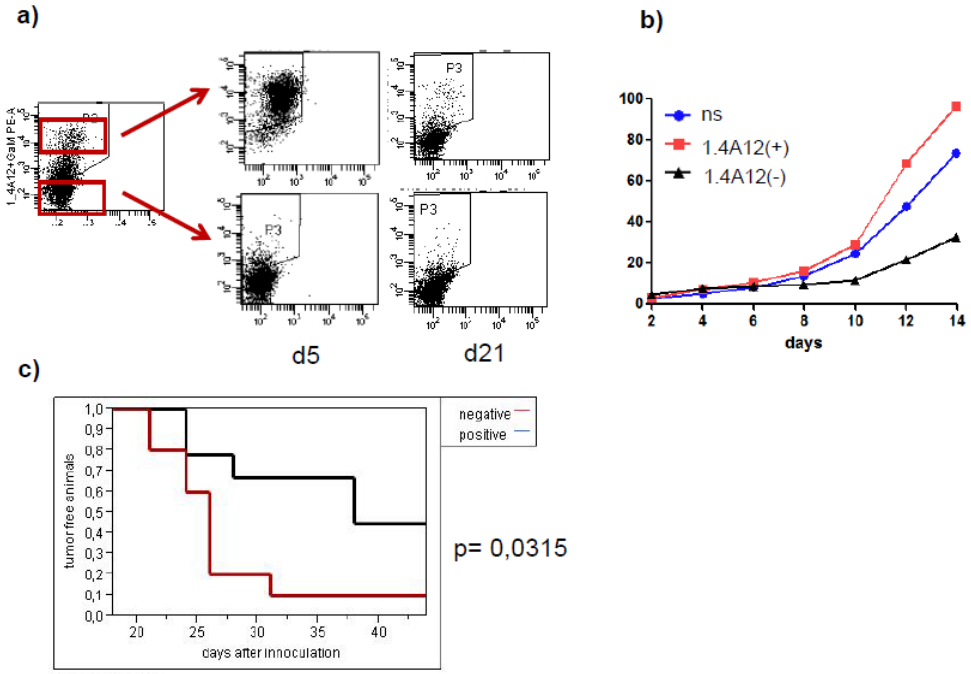


Figure 7: The ITGA7 positive population of SH-SY5Y cells is enriched for tumorigenic cells

a) SH-SY5Y cells were FACS sorted for ITGA7 positive and negative subpopulations. The expression of ITGA7 was controlled 5 and 21 days after the initial sorting by flowcytometry using the 1.4A12 antibody. **b)** Nonsorted (ns), 1.4A12 (ITGA7) positive (+) and negative (-) SH-SY5Y were seeded at a density of 1000 cells/well in a 96 well plate and the cell proliferation was determined every two days using an ATP-based assay (cell titer GLO). Plotted is the fold increase of ATP content compared to day 0. **c)** $5x10^4$ SH-SY5Y positive or negative for ITGA7 expression were subcutaneously injected in CD1/nude mice. The tumor development was monitored over a period of 50 days. The Kaplan-Meier plot shows the relative proportion of tumor free mice.

Primary BTSCs cells positive for integrin alpha 7 have clonogenic capacity

We sorted three different BTSC lines BTSC30pt, BTSC83TW and GBM T1 for integrin alpha 7 expressing high versus low levels of by flowcytometry. The cells were directly sorted into 96 well plates to 1 and 3 cells/ well and their clonogenic capacity was determined. The fraction of cells with high integrin alpha 7 expression showed a significantly higher clonogenic capacity in all three lines investigated (see figure 8). These results indicated an enrichment of potentially tumor initiating cells in the integrin alpha 7 positive population of primary glioblastoma stem like cells and showed a possible role as marker for the aggressiveness of primary BTSCs.

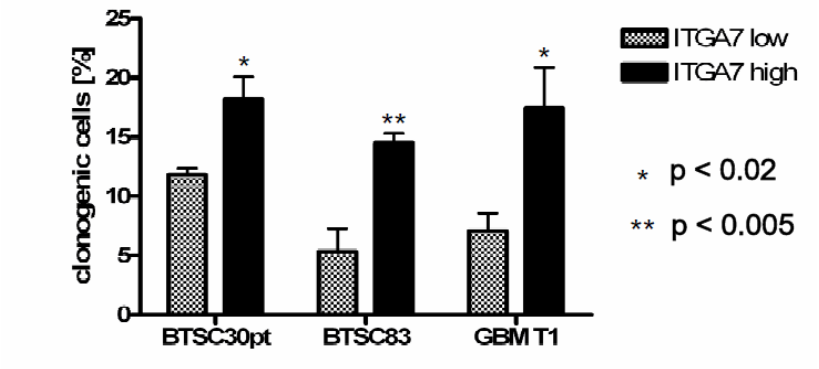


Figure 8: The ITGA7 positive population of primary BTSC cells is enriched for clonogenic cells

a) Three glioblastoma stem cell lines (BTSC30pt, BTSC83 and GBM T1) were FACS sorted for ITGA7 positive and negative subpopulations. ITGA7 highly positive (high) and ITGA7 dim (low) cells were seeded at densities of 1 and 3 cells/well in a 96 well plate. The clonogenic capacity was determined 4 weeks after the plating. The wells containing clones were counted and the percentage of clonogenic cells was calculated. Plotted is the average and SEM of 3-4 independent laydown experiments performed by flowcytometry.

Integrin alpha 7 is crucial for proliferation and clonogenic survival of BTSC1 in vitro

To verify that integrin alpha 7 supported tumor cell growth we neutralized integrin alpha 7 expression by infecting BTSC1 cells with lentiviral particles encoding for small hairpin (sh) RNA, targeting the cDNA of integrin alpha 7. FACS analysis confirmed a substantial knockdown of integrin alpha 7 in cells treated with ITGA7shRNA compared to the cells treated with control shRNA (not shown). We performed cell proliferation and clonogenicity assays with BTSC1 and BTSC83 cells knocked down for integrin alpha 7. We detected a highly significant decrease in cell growth in cells with reduced integrin alpha 7 levels (figure 9a and c). Even more evident was the reduction of the clonogenic survival of the cells treated with integrin alpha 7 shRNA, which was almost completely blunted, while up to 30% of the cells transduced with control shRNA were clonogenic (figure 9b and d). We performed the silencing of ITGA7 with 2 additional primary BTSC lines (BTSC 30pt, BTSC151) and GBM T1, a very low passage (P3-5) spheroid culture from freshly isolated glioma cells. All the lines shown a significant reduction in proliferation, when treated with both integrin alpha 7 targeting shRNA constructs (figure 9e-g). Taken together these results clearly underlined an essential role for integrin alpha 7 for proliferation of primary glioblastoma stem cells *in vitro* independently from the cellular system used.

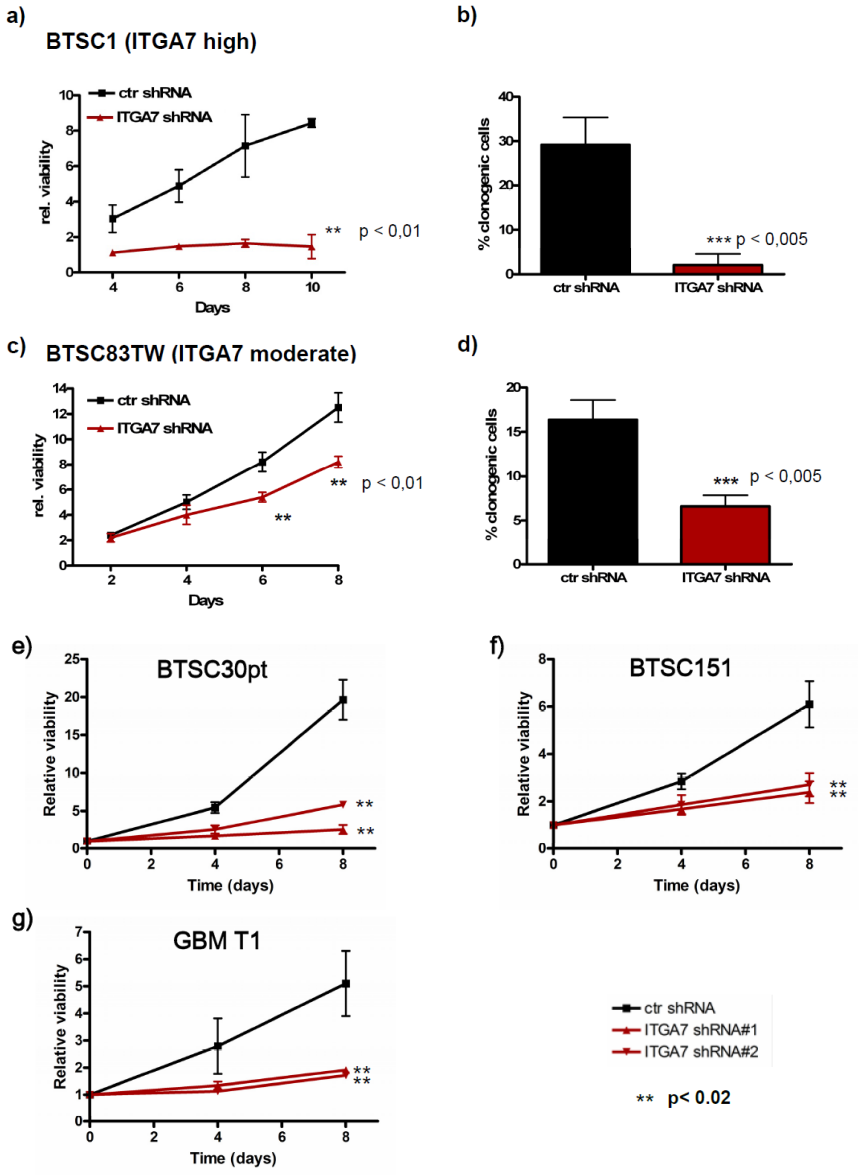


Figure 9: ITGA7 is crucial for the growth and clonogenicity of BTSC1 *in vitro*

a)BTSC1 cells were infected with lentiviral particles either encoding for a control small hairpin RNA (ctr-shRNA) or for shRNA targeting ITGA7 expression. Cells were seeded at densities of 500 cells/well in a 96 well plate and the cell proliferation was determined at the indicated time points using an ATP-based assay (cell titer GLO). Plotted is the fold increase of ATP levels compared to day 0. Shown are average and SEM of 3 independent experiments. (Student t test: **p< 0,01, ***p<0,005). **b)** Cells treated like in (a) were seeded at a density of 1 cell/well in 96 well plates. 21 days after plating the wells were controlled for colonies and shown are average and SD of colonies detected in 4 independent experiments. **c)** and **d)**: same experiment as described in (a) and (b) using a second primary brain tumor stem like cell line (BTSC83). **e-g**): Growth curves as in (a) performed with 3 additional primary glioblastoma stem like cells. ITGA7 was knocked down using two independent shRNA constructs. Shown are average and SEM of 3 independent experiments. (Student t test: **p< 0,02)

Integrin alpha 7 mediates cell cycle progression in primary brain tumor stem cells

To analyze the molecular mechanism of integrin alpha 7 involvement in tumor cell progression we performed the western blot analysis for proteins implicated in cell cycle regulation. We observed a marked decrease in phosphorylated retinoblastoma (RB) protein, p21, Cyclin B1 and Polo like kinase 1 (PLK1) in cells knocked down for integrin alpha 7 expression, in both BTSC lines investigated, BTSC1 and BTSC83 (figure 10 a). The biochemical evidence for the block in cell cycle progression was confirmed by flowcytometric cell cycle analysis, showing for both lines an accumulation of nuclei in G1 phase (figure 10 b-d). These results suggested, that integrin alpha 7 is crucial for the aberrant cycle progression of primary glioblastoma stem like cells and that interfering with integrin alpha 7 function might be a powerful tool to interfere with tumor growth.

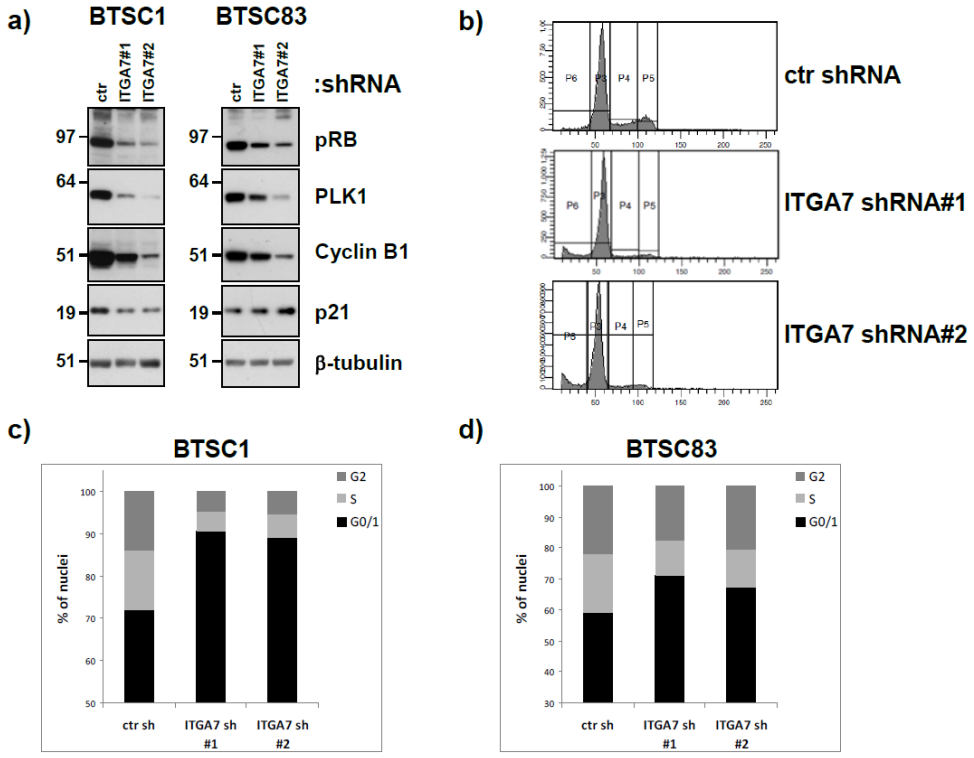


Figure 10: ITGA7 knockdown leads to cell cycle arrest in glioblastoma stem like cells

a) Shown is a western blot analysis of BTSC1 and BTSC83 cells either transfected with control shRNA or two independent ITGA7 targeting shRNAs. The membranes were probed with the antibodies recognizing cell cycle dependent proteins indicated. **b)** BTSC1 cells were infected with lentiviral expression constructs for ctr shRNA or two independent shRNAs targeting ITGA7. 9-11 days after transduction cell cycle analysis was performed. Shown are representative histograms obtained by flowcytometry. **c)** Represents the analysis of the cell cycle analysis described in (b). **d)** Shows the block in cell cycle progression upon ITGA7 knockdown in a second glioblastoma stem like cell line (BTSC83)

Integrin alpha 7 is crucial for proliferation and clonogenic survival of BTSC1 in vivo

After the clear evidence of anti-proliferative effect upon the silencing of integrin alpha 7 *in vitro*, we transfered the system in preclinical *in vivo* immune deficient mice (NSG mice) model systems. We found that the cells knocked down for integrin alpha 7 expression were significantly less tumorigenic when compared with the cells transduced with the control shRNA construct in a subcutaneous tumor model (figure 11). The results were very evident using BTSC1 cells, which showed a high expression of integrin alpha 7. These cells formed perceptible tumor nodules significantly later, when integrin alpha 7 expression was suppressed (figure 11a). More importantly only one out of 6 mice showed limited tumor growth 5 months after engraftment. In contrast the control cells readily formed small nodules and developed big tumors with 100% efficiency (figure 11b). Using BTSC83 cells, which display a lower surface expression of integrin alpha 7, we also detected a significant delay in engraftment, even though 100% of the mice injected developed tumors (figure 11c). the tumor growyh of cells in which expression of ITGA7 was suppressed by shRna was slower compared with the control cells (figure 11d).

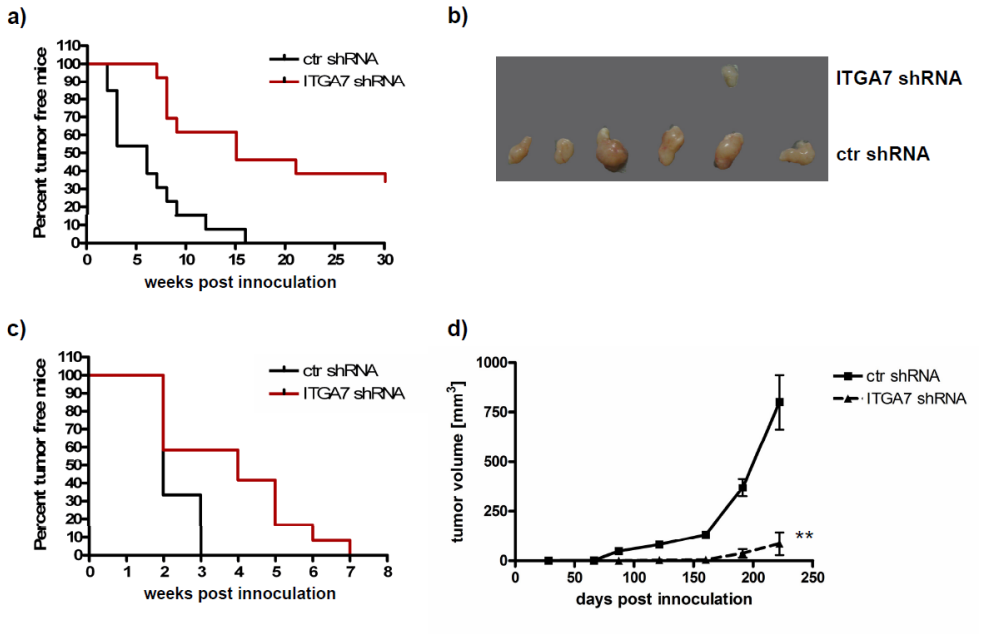


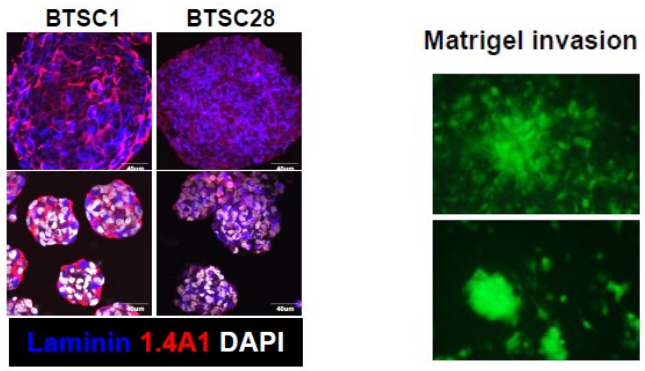
Figure 11: ITGA7 knockdown impairs tumor growth in xenograft model

a) Shown is the formation of palpable nodules upon injection of 100000 BTSC1 either transduced with control shRNA (ctr shRNA) or shRNA targeting ITGA7. **b)** BTSC1 tumors were explanted 30 weeks post inoculation. Shown is the harvest of one out of two cages. **c)** Formation of palpable nodules upon injection of 100000 BTSC83 either transduced with control shRNA (ctr shRNA) or shRNA targeting ITGA7. **d)** growth curve of engrafted BTSC83 transduced with ctr or ITGA7 shRNA. ** indicate a p value of < 0.02 (two tailed student t test)

Integrin alpha 7 knockdown prevents laminin signaling and invasion of GBM cells in vitro

By IF analysis on BTSC in spheroid culture, we detected a coexpression of integrin alpha 7 and laminin in the spheres using a anti-laminin antibody (figure 12 a). This indicate that the spheroid culture seems to mimic the microenvironment in the primary tumor. We tested integrin alpha 7 might involvement on cell spread and invasion in vitro. We observed that compared to the control cells, cells knocked down for integrin alpha 7 spread much less into the matrigel (figure 12b) indicating a less invasive phenotype. To analyze the invasive capability of the cells, we performed an invasion assay using laminin coated transwells and we detected a highly significant reduction of invasion after 48h from the seeding in cells knocked down for integrin alpha 7 (figure 12c). We investigated the intracellular pathways involved in integrin signaling. FAK was readily phosphorylated when the cells were seeded on laminin coated plates (figure 13a). This effect was completely blunted by the knockdown of integrin alpha 7 using two independent shRNA constructs in different cell lines (figure 13b-d).

a) **b)**



c)

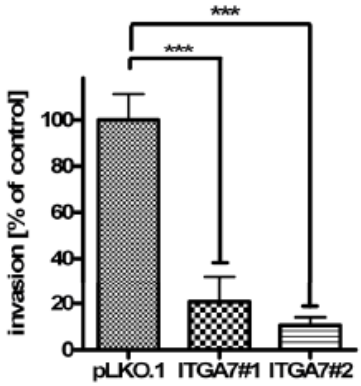


Figure 12: Integrin alpha 7 knockdown prevents laminin signaling and invasion of GBM cells in vitro

a) IF on spheres from two different primary spheroid cultures of GBM stained with the indicated antibodies. **b)** Matrigel invasion assay of BTSC1 spheres infected with lentiviral pLKO.1 ctr (upper panel) or a construct for the silencing of ITGA7(lower panel). The cells were also infected with a GFP containing vector for better visualization of the spreading. **c)** Invasion of BTSC1 in transwell chambers (BD fluoroblok) coated with laminin (10 µg/ml). After 48 hrs the cells were stained with Calcein AM. Shown are average and SEM of 3 independent experiments done in triplicate. *** indicate a p value of < 0.001 (ANOVA).

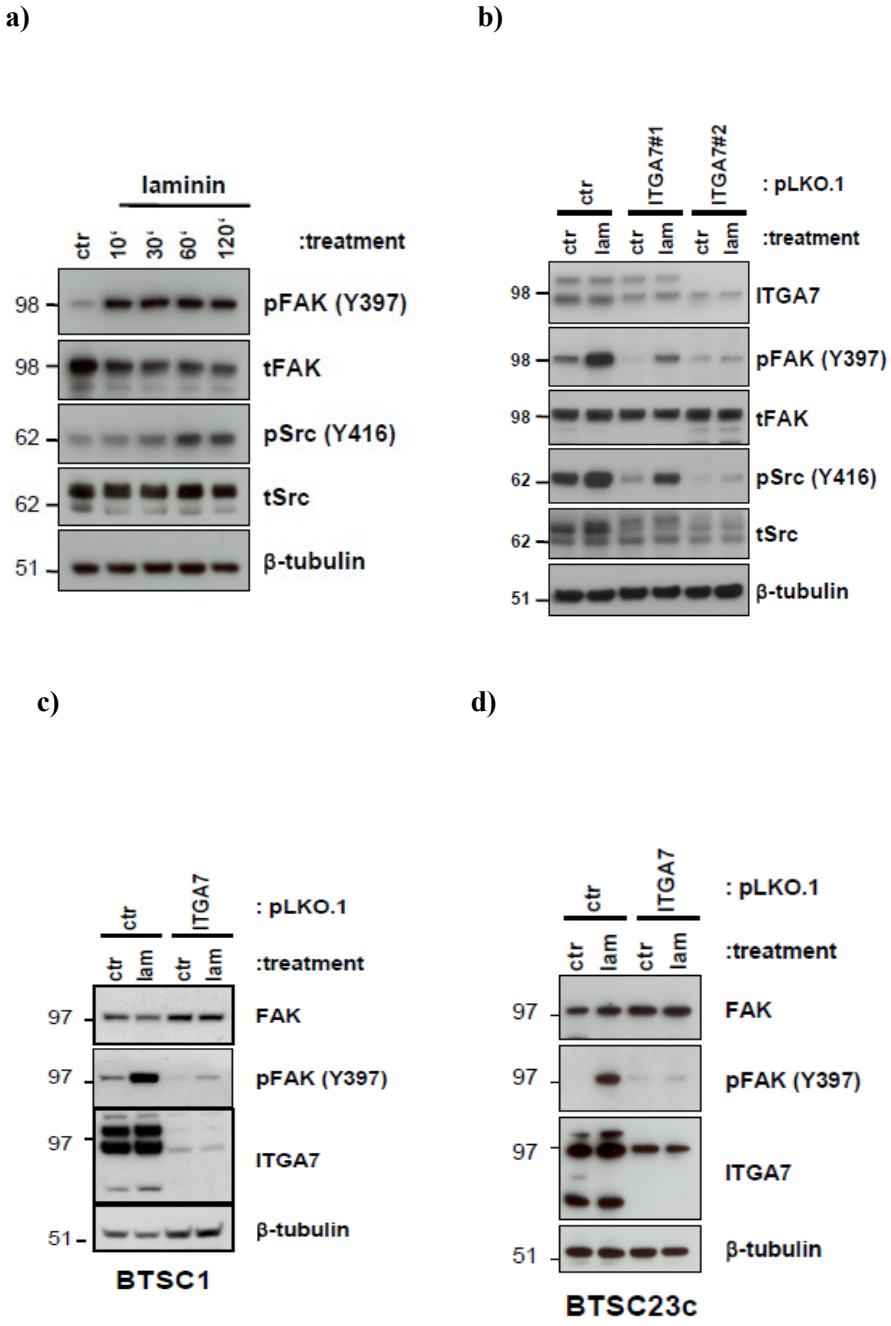
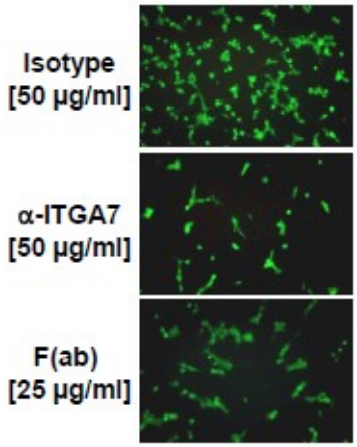


Figure13: a) BTSC1 cells were incubated on laminin coated plates for the time frames indicated. Then cells were lysed and western blot analysis for the indicated proteins was performed. **b)** BTSC1 cells treated with ctr or ITGA7 shRNA containing lentiviral particles were incubated on laminin coated plates for 60 min. Afterwards the cells were lysed and western blot analysis was performed. **c)** BTSC23p cells treated with as above. **d)**BTSC23c cells as above. Afterwards the cells were lysed and western blot analysis was performed

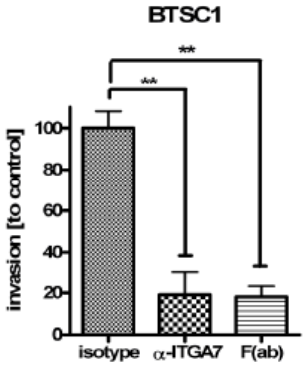
Anti integrin alpha7 antibody suppresses tumour growth in vitro and in vivo

Identified its potential role in GBM invasion, we characterized the biological function of the antibody which was generated in the initial step of our study. We performed the invasion assay with different primary spheroid GBM cultures and we found a strong inhibition of the invasive capability by treating the cells with the intact antibody and also with F(ab) fragment generated from 1.4A12 (figure 14a-b). Importantly the mAb 1.4A12 was capable to block the signalling of laminin. As described for the integrin alpha 7 knockdown, the cells pre-treated with 1.4A12 showed a massive reduction in FAK and Src phosphorylation when compared to non treated cells or cells pretreated with an isotype control antibody (figure 14 c-f). These results showed a blocking function of 1.4A12 with regards to cell invasion and laminin induced signaling in different cell lines used. To test a potential therapeutic value of this agent, we subcutaneously injected and treated the mice with two weekly doses of 10 mg/kg anti integrin alpha7 antibody (figure 15a). While shortly after implantation the luciferase activity was equal to the control group (figure 15b), at later time points the tumor engraftment was significantly impaired in the mice treated with the integrin alpha7 antibody (figure 15c). These results strongly suggested the potential therapeutic value of antibody mediated integrin alpha7 targeting *in vivo*.

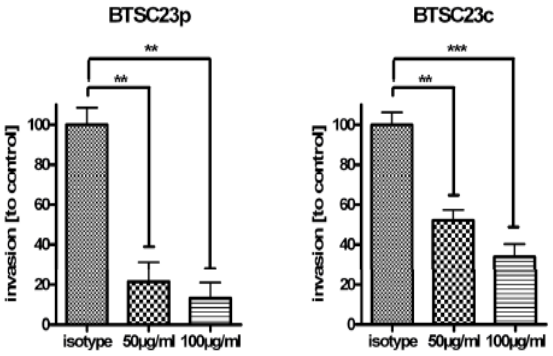
a)


Isotype
[50 µg/ml]
α-ITGA7
[50 µg/ml]
F(ab)
[25 µg/ml]

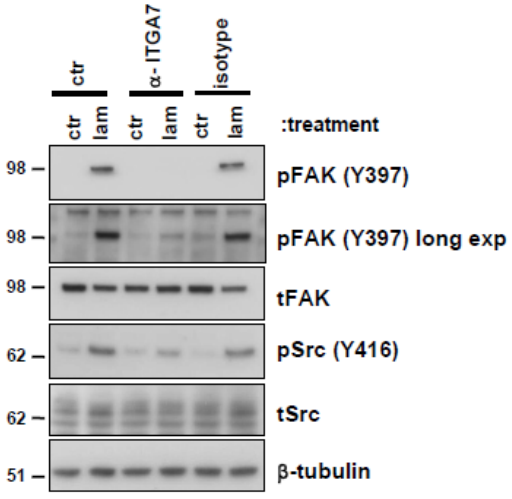
b)


BTSC1
**
**
invasion [to control]
100
80
60
40
20
0
isotype
α-ITGA7
F(ab)

c)


BTSC23p
**
**
invasion [to control]
100
80
60
40
20
0
isotype
50µg/ml
100µg/ml
BTSC23c
***
**
invasion [to control]
100
80
60
40
20
0
isotype
50µg/ml
100µg/ml

d)


ctr
α- ITGA7
isotype
ctr
lam
ctr
lam
ctr
lam
:treatment
98
pFAK (Y397)
98
pFAK (Y397) long exp
98
tFAK
62
pSrc (Y416)
62
tSrc
51
β-tubulin

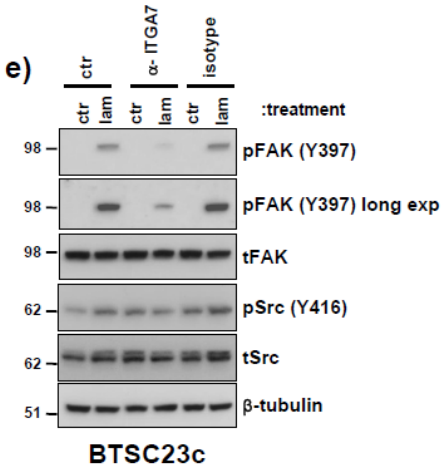


Figure 14: a) Invasion of BTSC1 in transwell chambers (BD fluoroblok) coated with laminin (10 µg/ml). After 48 hrs the cells were stained with Calcein AM. Shown is a picture with a representative result with BTSC1 cells.The photographic pictures of the migrated cells shown in **b)** were then analyzed with ImageJ as described in M+M. Shown are average and SEM of 3 independent experiments done in triplicate. ** indicate a p value of < 0.01 (ANOVA) **c)** Invasion of BTSC23p/BTSC23C in transwell chambers (BD fluoroblok) coated with laminin (10 µg/ml) in presence or absence of 1.4A12 (anti ITGA7) antibody. After 48 hrs the cells were stained with Calcein AM and the invaded cells were analyzed with ImageJ as described in M+M. Shown are average and SEM of 3 independent experiments done in triplicate. ** indicate a p value of < 0.01 (ANOVA). **d)** BTSC1 cells were preincubated with CSC medium either containing anti ITGA7 (1.4A12), non binding isotype control antibody or PBS for 15 min at 37°C. Then the cells were seeded on laminin coated plates for 30 min. Afterwards the cells were lysed and western blot analysis was performed. The membrane was stripped after every detection with the antibodies indicated. e) Western blot of BTSC23c treated as described above

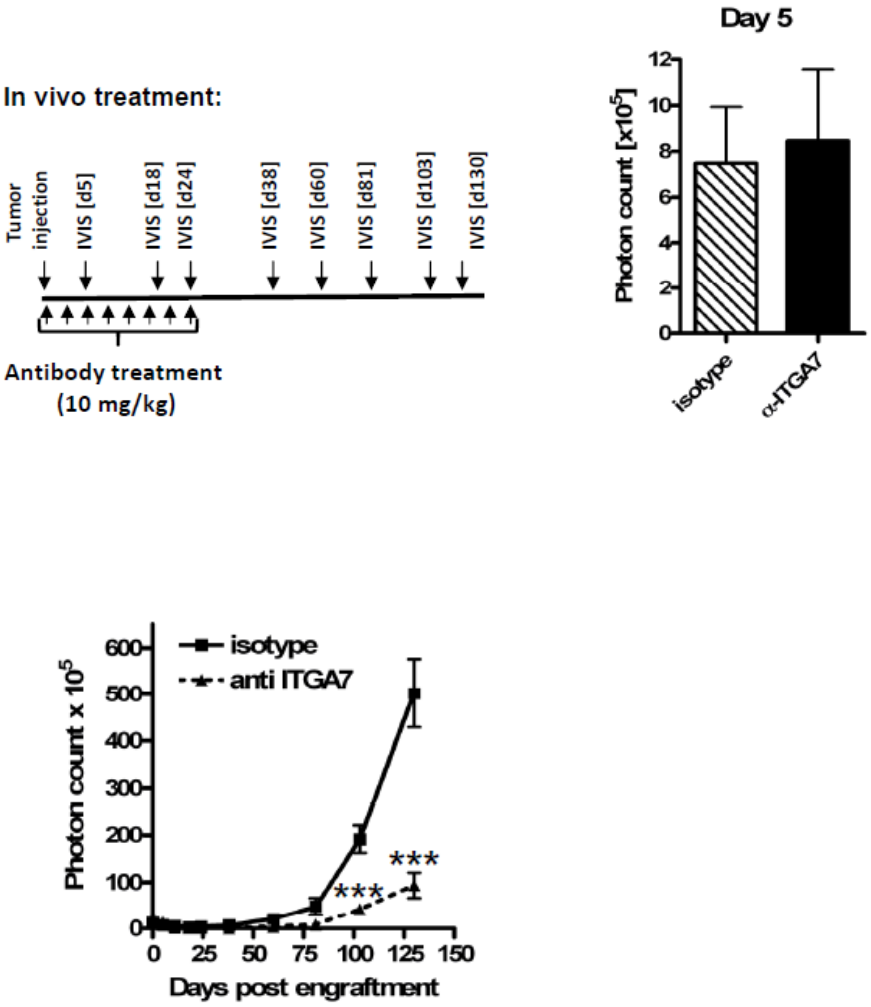


Figure 15: a) Treatment schedule of the in vivo treatment of BTSC1 with 1.4A12 anti ITGA7 antibody. **b)** Luciferase activity detected with a Xenogen IVIS 100 small animal in vivo imaging system, 5 days post s.c. transplantation of $5x10^5$ tumour cells in mice either treated with anti ITGA7 antibody (1.4A12) or isotype control. **c)** Analysis of tumour growth over time in mice treated either with isotype control, or with neutralizing anti ITGA7 antibody (1.4A12). Shown is the average photon count and SEM of 6 mice/group as measured by Xenogen IVIS 100 small animal in vivo imaging system *** indicate a p value of < 0.001 (two tailed student t

DISCUSSION

In this last decade oncology researcher focused their attention on the identification of new specific targets such as smart molecules tyrosine kinase specific (TK) inhibitors (Tol and Punt) or monoclonal antibodies to potentiate the standard therapy. Among the new target integrins have been involved in tumor progression for their ability to cooperate with growth factor receptors. Preclinical studies demonstrated that integrin antagonists inhibit tumor growth by affecting tumor cells and their microenviroment. Integrin antagonists used in clinical trials include monoclonal antibodies and RGD peptide mimetics (Avraamides, Garmy-Susini et al. 2008). Integrin αvβ3 (lM609) was found to be upregulated in both tumor cells and endothelial cells. Blocking monoclonal antibody against Integrin αvβ3 in some patients with advanced prostate solid tumors (Delbaldo, Raymond et al. 2008) and renal cancer (McNeel, Eickhoff et al. 2005) showed considerable anti-angiogenic activity in preclinical studies (Brooks, Stromblad et al. 1995). Its humanized version , Etaracizumab shown anti-angiogenic effects, reduced tumor growth (Mulgrew, Kinneer et al. 2006), low toxicity, disease stabilization and impaired bone resorption by inhibiting osteoclast attachment, suggesting possible efficacy in reducing bone metastasis (Gramoun, Shorey et al. 2007). Etaracizumab was one of the first integrin antagonists introduced into clinical trials. Other example is the human αv integrin specific monoclonal antibody CnTo95, which targets both αvβ3 and αvβ5 integrins, also had anti-tumor and anti-angiogenic effects in xenograft tumor models (Trikha, Zhou et al. 2004) and in a Phase I trial was non-toxic, localized to tumors and showed anti-tumor activity (Mullamitha, Ton et al. 2007). Cilengitide, cyclic RGD peptides inhibitor for αvβ3 and αvβ5 integrins, (Smith, Ruggeri et al. 1990), is in Phase II trials in patients with lung and prostate cancer (Beekman, Colevas et al. 2006) and in Phase II and Phase III trials for glioblastoma treatment. Cilengitide has been shown promising results in patients with late-stage glioblastoma by extending patient survival with minimal side effects (Reardon, Fink et al. 2008). Cilengitide has an anti-angiogenic activity and affects multiple cell types in the tumor microenvironment, including the tumor cells. In glioblastomas, that is a tumor highly vascularized, the expression of integrin αvβ3 on angiogenic blood vessels, as well as the tumor cells themselves, suggest that antagonists to this integrin might be therapeutically beneficial in patients with glioblastoma. Infact in preclinical studies, cilengitide effectively inhibited angiogenesis and the growth of orthotopic glioblastoma (Yamada, Bu et al. 2006). High-grade glioblastomas express the ECM protein vitronectin, an integrin αvβ3 ligand, and this interaction affects

tumor cell survival and invasion. Therefore, the relatively large quantity of vitronectin present in the brain microenvironment, surrounding glioblastomas, might explain why these tumors are susceptible to the treatment with anti integrin antibodies. Phase I studies with cilengitide in patients with recurrent glioblastoma showed that it was well tolerated and produced durable responses (Nabors, Mikkelsen et al. 2007).

Several monoclonal antibodies are under evaluation in early-phase clinical trials in combination with RT and temozolomide for the treatment of GBM such as cetuximab and erlotinib which inhibit EGFR (Van den Bent et al, 2009). PDGFR amplification and overexpression are common in secondary GBM. Phase II studies have evaluated imatinib, the best-known PDGFR inhibitor, in recurrent GBM as a single agent (Wen et al, 2006) and in combination with hydroxyurea (Reardon et al, 2005). Multiple c-MET inhibitors are currently under evaluation such as AMG102, a human monoclonal antibody in phase II studies.

The aim of our study was to generate monoclonal antibodies (mabs) against Cancer Stem Cells (CSC) isolated from glioblastoma multiforme patients (GBM). To produce new antibodies against GBM, we used immunocompetent BALB/c mice and immunized them with intact BTSC1 (brain tumor stem cell clone 1). We generated hybridomas and the supernatants from each well, that showed the growth of clones, was screened by high throughput screening FACS analysis (HTS FACS) simultaneously for reactivity towards the BTSC1 used for immunization, the in vitro differentiated BTSC1 and a pool of human normal white blood cells to exclude those with a broad anti human reactivity. After this screening we focused our attention on the antibodies that preferentially binds BTSC lines and not differentiated brain tumor cell lines and the lymphocytes. The hybridomas have been subcloned and the monoclonal antibodies (mAbs) purified by affinity HiTrap HP Protein A or Protein G column, depending on the mAb isotype. We verified the results obtained in the initial screening by comparing the binding of 5 hybridoma candidates on the surface of 4 couples of stem-like and serum differentiated BTSC clones. We selected the hybridoma 1.4A12, which reproducibly showed a very abundant binding to most BTSC lines, while being much less reactive towards differentiated, ATCC derived, standard GBM lines. To characterize the localization of the 1.4A12 antigen in more detail, we performed immunofluorescence staining of BTSC spheres and differentiated cells using antibodies recognizing well accepted stemness and differentiation markers of GSCs. We proved the localization of the 1.4A12 almost exclusively on the membrane of BTSC. These results pointed towards a highly specific mAb and a potential new surface biomarker for GBM. The

specific protein was purified by immunoprecipitation from CSC lysate and analyzed by mass spectrometry in collaboration with the proteome analysis group of the German Cancer Research Center in Heidelberg. The mass spectrometric analysis revealed that 1.4A12 antibody pulls down integrin α7 (ITGA7). By performing FACS sorting experiments, we demonstrated that the ITGA7 high expressing population of the neuroblastoma cell lines SH-SY5Y had a higher growth rate in vitro and a significantly higher tumorigenic potential in vivo.

ITGA7 silencing has been shown to reduces tumor cell growth rate and clonogenicity in vitro. This growth reduction in tumor cell was observed in all the BTSC lines tested. Western blot analysis for the cell cycle related proteins showed a marked decrease in the proteins essential for proliferation in ITGA7 silenced cells. In vitro data where confirmed by in vivo experiments demonstrating that ITGA7 function interference leads to a tumor cell growth reduction in preclinical animal model. This demonstrated that integrin alpha7 plays an important role in tumor growth.

In literature is well reported that the integrins are involved in tumor invasion and tumor progression (Guo and Giancotti 2004). To investigate the potential integrin alpha7 involvement in cell invasion, we demonsctrated that ITGA7 silencing markedly reduced the invasion of BTSC in vitro by performing transwell-based laminin invasion assays. The antibody recognizing integrin alpha7 (mAb 1.4A12), interferes with integrin alpha 7 protein tumorigenic function in GBM. Performing a large panel of fluoroblok-based invasion assays, we demonstrated that mAb 1.4A12 treatment inhibited the invasion on laminin coated plate. These results underline the importance of 1.4 antibody to block the invasive signaling. Western blots analysis showed that the ITGA7 silencing or the treatment with mAb 1.4A12 blocks FAK activation that is the initial mediator of the integrin signal pathway (Pu and Streuli 2002). We demonstrated for the first time a biological function of integrin alpha7 in glioblastoma stem cells and the therapeutic potential value of anti-ITGA7 mAb in subcutaneous xenograft models giving the basis for preclinical studies in glioblastoma treatment.

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