UNIVERSITA' DEGLI STUDI DI UDINE

TESI DI DOTTORATO DI RICERCA

ISOLATION AND CHARACTERIZATION OF STEM CELLS ISOLATED FROM HUMAN LOW-GRADE AND HIGH-GRADE GLIOMAS

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ANNO ACCADEMICO 2011/2012

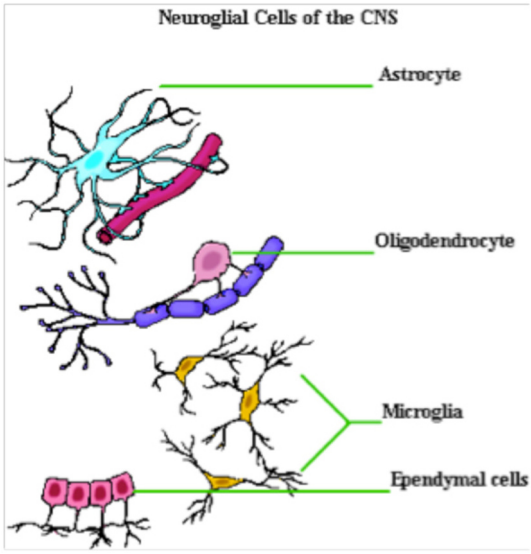
INTRODUCTION

1.1 Anatomy of the Glia

Glioma is the general term used to describe any tumor that arises from the supportive tissue of the brain. It is called *glioma* because it arises from glial cells (also called neuroglia; Figure 1.1 and Figure 1.2) that provide physiological support to neurons and repair neuronal damage due to injury or disease.

It is demonstrated that glial cells:

1. Provide mechanical support to neurons.
2. Because of their non-conducting nature, glial cells act as insulators between neurons and prevent neuronal impulses from spreading in unwanted directions
3. They can remove foreign material and cell debris by phagocytosis.



4. They can repair damaged areas of the nervous tissue by proliferation (gliosis), as they form glial scar tissue, and fill the gaps left by degenerated neurons.
5. Glial cells can take up and store neurotransmitters released by the neighboring synapses. Neurotransmitters can either be metabolized or released again from the glial cells.
6. They help in neuronal functions by maintaining a suitable metabolic and ionic environment for the neurons.

Figure 1. 1. Neuroglial cells of the CNS. From http:// www.anatomy.com/basic-anatomy/glial-cells-neuroglia

7. Ependymal cells are involved in the exchanges between the brain and the Cerebrospinal Fluid.

The glial cells located in the parenchyma of brain and spinal cord are broadly classified as:

Microglia, of mesodermal origin;

Macroglia, of ectodermal origin, comprising astrocytes, oligodendrocytes and glioblasts.

Microglia: These are the smallest of the glial cells, characterized by a flattened cell body with a few short, fine processes. They are often related to capillaries, and are considered phagocytic in nature. Microglial cells are possibly derived from the circulating monocytes, which migrate into the Central Nervous System (CNS) during the late fetal and early postnatal life. However, the developmental origin of microglia remains debatable (5), the two major views affirm that they derive either from neuroepithelial cells (6, 7) or from hematopoietic cells (8, 9).

Macroglia: it's mainly composed by astroglia and oligodendroglia, and it is generally considered developmentally distinct from microglia, being derived from neuroectoderm (10).

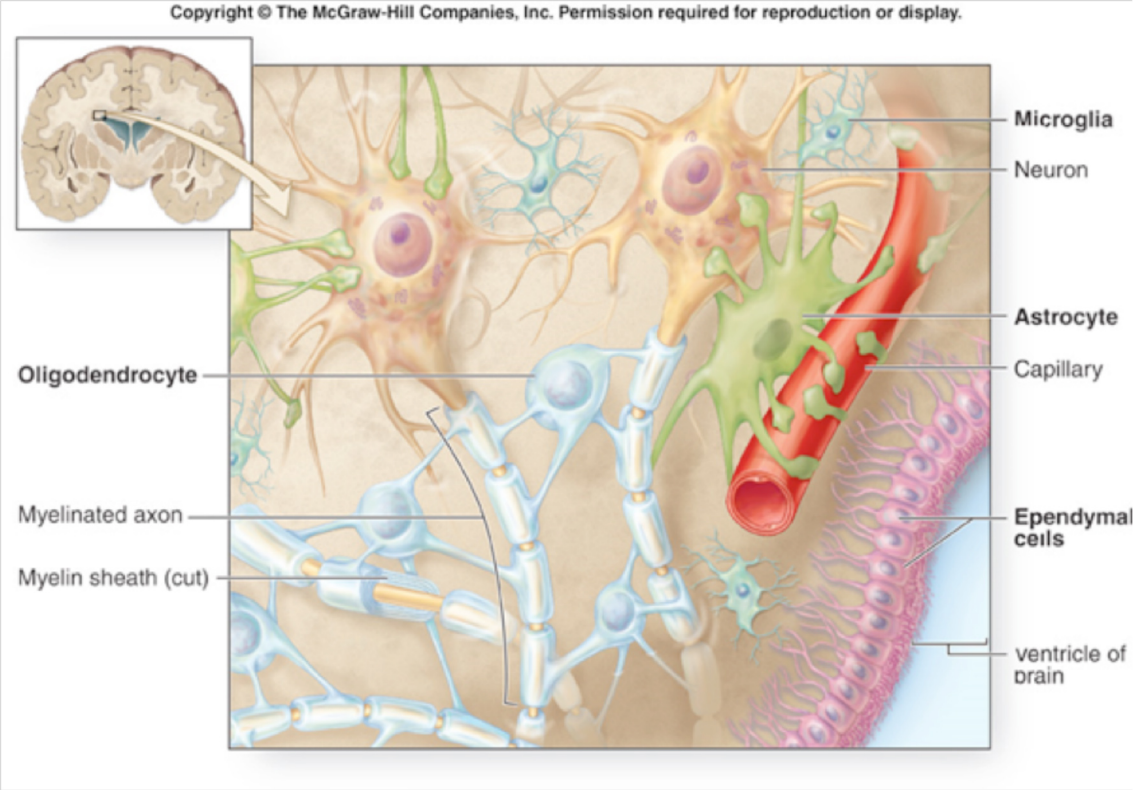


Figure 1. 2. Glial cells and their interactions with neurons. From: Biology of Oligodendrocyte and Myelin in the Mammalian Central Nervous System (7).

Astrocytes

Astrocytes have been divided into two main subtypes, *protoplasmic* or *fibrous*, on the basis of differences in their cellular morphologies and anatomical locations (11):

Protoplasmic astrocytes are found throughout all gray matter and, as first demonstrated using classical silver impregnation techniques, exhibit a morphology of several stem branches that give rise to many finely branching processes in a uniform globoid distribution;

Fibrous astrocytes are found throughout all white matter and exhibit a morphology characterized by many long fiber-like processes. Classical and modern neuroanatomical studies also indicate that both astrocyte subtypes make extensive contacts with blood vessel.

Oligodendrocytes

The term *oligodendroglia* was introduced by Del Rio-Hortega and collaborators to describe those neuroglial cells that, in material stained by metallic impregnation techniques, showed few processes (12). The oligodendrocyte is mainly a myelin-forming cell, but there are also satellite oligodendrocytes that may not be directly connected to the myelin sheath (13). Satellite oligodendrocytes are perineuronal and may serve to regulate the microenvironment around neurons (14, 15). A number of features consistently distinguish oligodendrocytes from astrocytes (15), in particular their smaller size, the greater density of both the cytoplasm and nucleus (with dense chromatin), the absence of intermediate filaments (fibrils) and glycogen in the cytoplasm, and the presence of a large number of microtubules (25 nm in diameter) in their processes that may be involved in their stability (16). An oligodendrocyte extends many processes, each of which contacts and repeatedly envelopes a stretch of axon with subsequent condensation of this multispiral membrane-forming myelin (15, 17).

Glioblast

Glioblasts are stem cells able to differentiate into macroglial cells. They are particularly numerous beneath the ependyma (18).

Ependymal cell

These neuronal supporting cells form the epithelial lining of the brain ventricles and of the central canal of the spinal cord. Ependymal cells also give rise to the epithelial layer that surrounds the choroid plexus, a network of blood vessels located in the walls of the lateral ventricles. Ependymal cells share, with all other neuroglial cells, a neuroectodermic origin (19).

1.2 Gliomas

As previously mentioned, gliomas, which comprise astrocytic, oligodendroglial, and ependymal lesions, are the most frequent primary intracranial tumors. This section will focus on glioma epidemiology, etiology, classification, histopathology, diagnosis and therapy.

1.2.1 Epidemiology and etiology of gliomas

Gliomas of astrocytic, oligodendroglial and ependymal origin account for more than 70% of all brain tumors (20, 21). The most frequent (65%) and most malignant histological type is the glioblastoma (20, 21). Since the introduction of computerized tomography and magnetic resonance imaging, the incidence rates of brain tumors have been rather stable, with a tendency of higher rates in highly developed, industrialized countries. Some reports indicate that Caucasians have higher incidence than black or Asian populations, but to some extent, this may reflect socio-economic differences and under-ascertainment in some regions, rather than a significant difference in genetic susceptibility.

In Italy, it is reported an incidence of 5-10 cases every 100.000 people, equally divided in both sex, even though most malignant forms are usually revealed in the male sex (20, 21). In the pediatric population, gliomas represent, after hematopoietic neoplasia, the most common class of tumors, with an incidence of 1-1,5 cases every 100.000 children aged from 0 to 14 years (20, 21).

With the exception of pilocytic astrocytomas, the prognosis of glioma patients is still poor. Less than 3% of glioblastoma patients are still alive at 5 years after diagnosis, higher age being the most significant predictor of poor outcome.

The *etiology* of gliomas is still not completely understood. However, different genetic elements seem to be implied as predisposing factors for the development of these pathologies. In particular, it has been reported the importance of p53 mutations (TP53) in low-grade gliomas (LGG) and secondary glioblastomas derived therefrom. Approximately 60% of mutations are located in the hot spot codons 248 and 273, and the majority of these are G:C-->A:T transitions at CpG sites. TP53 mutations are significantly more frequent in low-grade astrocytomas with promoter methylation of the O(6)-methylguanine-DNA methyltransferase repair gene, suggesting that, in addition to deamination of 5-methylcytosine, exogenous or endogenous alkylation in the O(6) position of

guanine may contribute to the formation of these mutations. Conversely, the loss of heterozigosity (LOH) of chromosome 19 has been described in anaplastic astrocytoma (20, 21).

Yet, some epidemiologic studies underline the significant association between some hereditary syndromes (such as Type 1 and 2 neurofibromatosis and Li Fraumeni Syndrome) and the development of astrocytoma (20, 21)

Today, the only environmental factor unequivocally associated with an increased risk of brain tumors is exposure to ionizing radiations, including therapeutic X-irradiation. In particular, children treated with X-irradiation for acute lymphoblastic leukemia show a significantly elevated risk of developing gliomas and primitive neuroectodermal tumor (PNET), often within 10 years after therapy.

Other studies, although not conclusive, suggest cerebral traumas, nitrosamine rich food ingestion and electromagnetic fields exposure (cell phones predominantly) as possible predisposing factors (20, 21).

The association between occupations carcinogens and glioma occurrence has been object of different studies, demonstrating an elevated risk of astrocytoma in electric and electronic employees, proportional to the time of exposure. An increased risk has been found even in workers who have been exposed to organic chemicals in chemical and oil industries. Other possible carcinogens factors are tetrachloride carbon, tetrachloroetylene, trichloroetylene, but mostly cloride metylene (20, 21)

1.2.2 Glioma classification

The WHO classification, which incorporates the criteria of the St. Anne/Mayo criteria, is usually recommended as a recent and updated international standard for classifying and grading gliomas (Table 1.1)(22).

This classification is based on the premise that each type of tumor results from the abnormal growth of a specific cell type and includes criteria for their grading(22).

Therefore, according to WHO, gliomas are:

- Divided on the basis of morphologic criteria (cell shape, cytoplasmic appearance and the character of the nuclei) into astrocytomas, oligodendrogliomas, and oligoastrocytomas;
- Classified into 4 grades, according to their increasing malignancy, from grade I to grade IV. The grading is based on histological criteria such as cellularity, nuclear atypia, vascularisation and necrosis.

Importantly, this grading system correlates well with the clinical prognosis and most named tumors are of a single defined grade (Table 1.1).

	I	II	III	IV
Astrocytic tumours				
Subependymal giant cell astrocytoma	•			
Pilocytic astrocytoma	•			
Pilomyxoid astrocytoma		•		
Diffuse astrocytoma		•		
Pleomorphic xanthoastrocytoma		•		
Anaplastic astrocytoma			•	
Glioblastoma				•
Giant cell glioblastoma				•
Gliosarcoma				•
Oligodendroglial tumours				
Oligodendroglioma		•		
Anaplastic oligodendroglioma			•	
Oligoastrocytic tumours				
Oligoastrocytoma		•		
Anaplastic oligoastrocytoma			•	
Ependymal tumours				
Subependymoma	•			
Myxopapillary ependymoma	•			
Ependymoma		•		
Anaplastic ependymoma			•	
Choroid plexus tumours				
Choroid plexus papilloma	•			
Atypical choroid plexus papilloma		•		
Choroid plexus carcinoma			•	
Other neuroepithelial tumours				
Angiocentric glioma	•			
Chordoid glioma of the third ventricle		•		
Neuronal and mixed neuronal-glial tumours				
Gangliocytoma	•			
Ganglioglioma	•			
Anaplastic ganglioglioma			•	
Desmoplastic infantile astrocytoma and ganglioglioma	•			
Dysembryoplastic neuroepithelial tumour	•			
Central neurocytoma		•		
Extraventricular neurocytoma		•		
Cerebellar liponeurocytoma		•		
Paraganglioma of the spinal cord	•			
Papillary glioneuronal tumour	•			
Rosette-forming glioneuronal tumour of the fourth ventricle	•			
Pineal tumours				
Pineocytoma	•			
Pineal parenchymal tumour of intermediate differentiation		•	•	
Pineoblastoma				•
Papillary tumour of the pineal region		•	•	
Embryonal tumours				
Medulloblastoma				•
CNS primitive neuroectodermal tumour (PNET)				•
Atypical teratoid / rhabdoid tumour				•
Tumours of the cranial and paraspinal nerves				
Schwannoma	•			
Neurofibroma	•			
Perineurioma	•	•	•	
Malignant peripheral nerve sheath tumour (MPNST)		•	•	•
Meningeal tumours				
Meningioma	•			
Atypical meningioma		•		
Anaplastic / malignant meningioma			•	
Haemangiopericytoma		•		
Anaplastic haemangiopericytoma			•	
Haemangioblastoma	•			
Tumours of the sellar region				
Craniopharyngioma	•			
Granular cell tumour of the neurohypophysis	•			
Pituicytoma	•			
Spindle cell oncocytoma of the adenohypophysis	•			

Table 1.1 WHO Grading of Tumours of the Central Nervous System(22)

On the basis of location, gliomas can be classified, according to whether they are above or below the tentorium, a membrane in the brain, which separates the cerebrum from the cerebellum, in (23, 24):

- Supratentorial gliomas (above the tentorium, in the cerebrum), mostly present in the adults (70%);

- Infratentorial glioma (below the tentorium, in the cerebellum), mostly present in children (70%).

From a clinical point of view, gliomas are further categorized, depending on the biologic aggressiveness, in (25):

- **Low-grade gliomas** (**LGG**): they usually comprehend WHO class I and class II, well differentiated, slowly growing gliomas, characterized by a better prognosis;
- **High-grade gliomas** (**HGG**): they comprehend malignant WHO class III and IV, rapidly growing gliomas embedded with a worse prognosis.

This categorization matches well epidemiological data, and it is considered fundamental in predicting prognosis, guiding therapy and directing follow-up. However, there are cases in which it is difficult to classify a lesion as HGG or LGG. Moreover, gliomas, especially LGG, are often characterized by a discrete clinical heterogeneity, thus making difficult to predict prognosis, and therefore decide the therapeutic approach, on the basis of the histological appearance. Therefore, there is a great need to implement the histological analysis with neuroradiological data, and evaluation of molecular abnormalities known to occur in these tumours(26).

1.2.3 Low-Grade Gliomas

The term Low-Grade Gliomas (*LGG)* includes all World Health Organization (WHO) grade I and II gliomas(27). Low-grade glioma (LGG) categories include subependymal giant cell astrocytoma, pilocytic astrocytoma, pilomyxoid astrocytoma, diffuse astrocytoma, pleomorphic xanthoastrocytoma, oligodendroglioma, oligoastrocytoma and certain ependymomas(28). LGG are most common among men and white people and typically affect patients at a younger age than high-grade gliomas (fourth versus sixth decade of life). LGG most commonly involve the cerebral hemispheres, and are typically located in the frontal, parietal, or temporal lobes.

LGG grow slowly but about 70% of grade II gliomas evolve to anaplasia within 5-10 years(29). Importantly, the natural course of LGG varies considerably and is highly influenced by treatment-independent factors, such as age, pretreatment performance score, tumor volume, contrast-enhancement on CT/MRI, and tumor histology(27). Therefore, the management of patients with LGG is a challenge, because: 1. There are not definitive criteria to classify a lesion as at high risk or low risk to relapse and/or to progress; 2. Many of the potential adjuvant treatments can produce or contribute to chronic neuro-cognitive function impairment, particularly radiotherapy; these side effects are not justifiable in patients that are possibly at low risk of relapse/progression2; 3. With the

exception of temozolomide (TMZ), current therapies are mainly designed according to previously tested molecules against other types of cancer; in fact, novel drugs specifically designed to target LGG are not yet available. Researches focused on other cancer types are currently exploiting these issues taking advantage of: 1. Wide genome analysis; 2. Drug discovery approach; 3. Identification of putative novel therapeutic targets within the tumor, such as tumor-initiating cells, tumor associated fibroblasts and infiltrating Mesenchymal Stem Cells (MSC).

For LGG all of these topics have been only incompletely explored. While a comprehensive genomic characterization defining human glioblastoma genes and core pathways is available (2, 30-34), this extensive analysis is missing for LGG. What we know is that, genetically, the vast majority of LGG are mutated in IDH1, frequently deleted in 1p19q (oligodendroglioma) or mutated in p53 (astrocytoma)(26, 35, 36). The IDH1 mutation is inversely correlated with grade, tightly associated with a 1p19q co-deleted genotype and a MGMT methylated status but mutually exclusive with Epidermal Growth Factor Receptor (EGFR) amplification and loss of chromosome 10(35, 36). Moreover, abnormalities in the PTEN tumor suppressor gene and the BRAF oncogene are under investigation(28).

1.2.4 High-Grade Glioma

High-grade gliomas comprise glioblastoma (WHO grade IV), anaplastic astrocytoma (WHO grade III), mixed anaplastic oligoastrocytoma (WHO grade III) and anaplastic oligodendroglioma (WHO grade III)(37).

Although **anaplastic astocytoma** can be diagnosed as a de novo tumor, in the majority of patients (50-75%) it represents a progression of pre-existing diffuse astrocytomas. This latter form affects mostly patients 35-55 years old, and it has an overall survival of nearly 36 months. The malignant progression of a diffuse astrocytoma, usually monitored by an enhancing in the contrast in Magnetic Resonance Imaging (MRI), is detected in 5 to 10% of the cases. Surgery is usually the initial therapeutic approach, but considering the infiltrative nature of anaplastic gliomas, it is difficult to completely eradicate the tumor. With respect to LGG, anaplastic gliomas are characterized by an increased cellular proliferation, nuclear pleomorphism, mitosis, glomerular endothelial proliferation, and necrosis.

From a genetic point of view, the mutation of TP53 is frequently found in anaplastic gliomas that evolve from low-grade glioma. To confirm the role of TP53 in the glioma's evolution, it's emphasized that in 90 % of the patients with this protein mutation in the tumor relapse, already presented TP53, mutation, in the primitive low-grade glioma.

Nevertheless, in anaplastic tumor there are many other abnormal conditions like deletion of the protein p16 (in 30% of the patients), alteration in the expression of the protein RB (in 25% of the cases), co-deletion of chromosome 1p/19q (in 15% of the cases), mutation in IDH1/2, as well as the tumor suppressor gene PTEN (15%) and finally the amplification in the Epidermal Growth Factor Receptor (EGFR) in 10%.

Besides that it has also been reported an increased loss of heterozygosity **(LOH)** regarding, in particular, the chromosome 10p (30-60%) and 19 (40%) as well as chromosome 22q (30%), along with the deletion of chromosome 6(33%).

The high incidence of the TP53 mutation and the elevated frequency of chromosomal aberrations compared to the low-grade glioma, indicates that anaplastic glioma is the transitional form between the low-grade glioma and glioblastoma multiform (secondary). Nevertheless the pathogenesis of patients with primitive anaplastic glioma is still unknown.

Glioblastoma multiforme (GBM) is the most common and aggressive form of malignant astrocytoma and can arise de novo or from pre-existing lower-grade tumors (22). The incidence of GBM in Italy is of 2-3 new cases every 100.000 people per year (27). GBM can occur at any age but is more likely to develop in older people (median age 53 years). It is predominantly found in male (27). Although in adults GBM develops mainly in the subcortical region, in the cerebral hemisphere and, especially, in the temporal lobe, in children it develops mostly in the cerebral trunk of the brain (38). It is generally associated with a poor prognosis (mean survival 11 months), yet individual patient survivals vary.

Historically, GBMs have been categorized into two groups: “primary” and ”secondary” GBM (39). The vast majority of glioblastomas (approximately 90%) develop rapidly de novo in elderly patients, without clinical or histological evidence of a less malignant precursor lesion (primary glioblastomas)(40). Secondary glioblastomas progress from low-grade diffuse astrocytoma or anaplastic astrocytoma. They manifest in younger patients, have a lesser degree of necrosis, are preferentially located in the frontal lobe and carry a significantly better prognosis. Histologically, primary and secondary glioblastomas are largely indistinguishable, but they differ in their genetic and epigenetic profiles (Figure 1.3). Decisive genetic signpost of secondary glioblastoma are IDH1 mutations(41), that are absent in primary glioblastomas and which are associated with a hypermethylation phenotype(40). IDH1 mutations are the earliest detectable genetic alteration in precursor low-grade diffuse astrocytomas and in oligodendrogliomas, indicating that these tumors are derived from neural precursor cells that differ from those of primary glioblastomas(40). According to Yan and other collaborators, the mutation of IDH1, promote the progression of low-grade glioma into high grade (42).

Clinical differences have been reported between the two groups, with secondary GBMs occurring predominantly in younger patients (45 years versus 60 years for primary GBM) (43, 44).

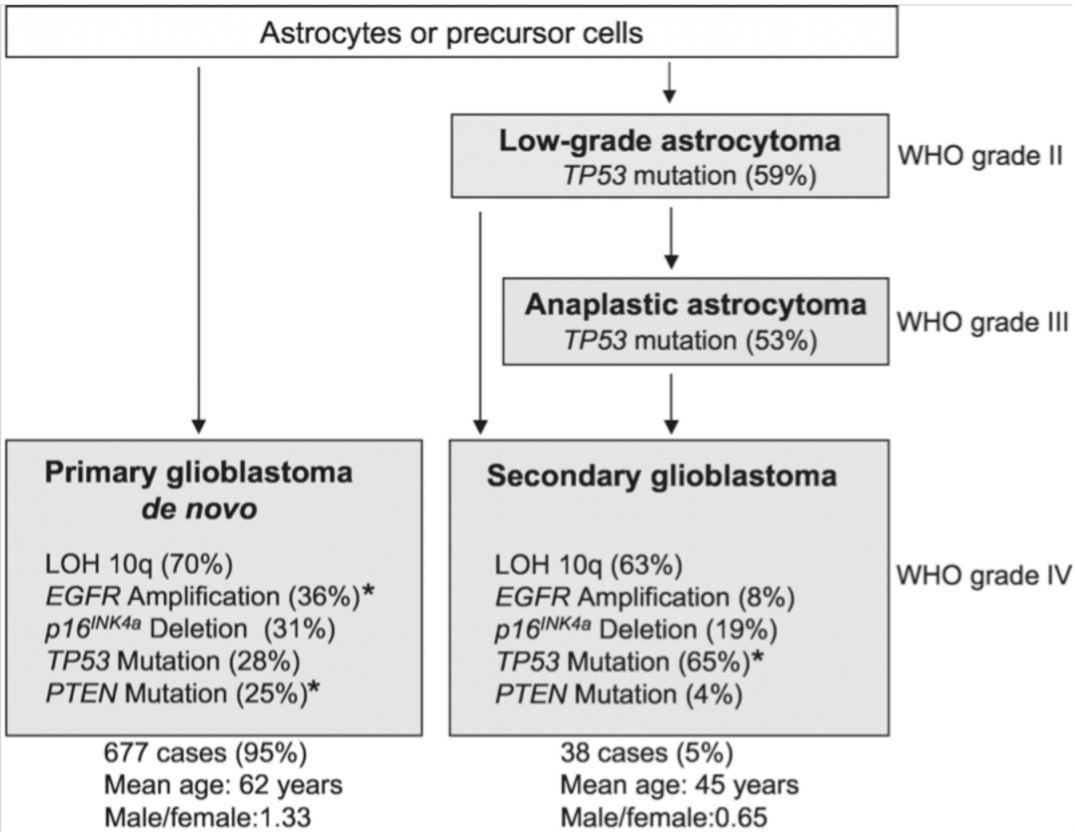


Figure 1. 3 Genetic alteration in primary and secondary GBM. From (42).

Recently, advances in molecular technologies, especially high-density microarray and genome sequencing, have made it possible to evaluate genetic and epigenetic changes in GBM at the genome-wide level(45). Specifically, these integrative genomic studies provided a comprehensive view of the complex genomic landscape of GBM, revealing a set of core signaling pathways commonly activated in GBM (Figure 1.4): the P53 pathway, the RB pathway, and the RTK pathway [reviewed in(45)]. The majority of GBM tumors have genetic alterations in all three pathways, which helps to fuel cell proliferation and enhance cell survival while allowing the tumor cell to escape from cell-cycle checkpoints, senescence, and apoptosis(45). Moreover, these studies have revealed the role of novel pathways, such as the involvement of NF1 mutation in 23 % of sporadic human GBM, the heterozygous deletion of the NF-kB inhibitor a (NFKBIA) gene in 25% of GBM samples, and the previously mentioned mutation of IDH1 in 12% of GBM(45).

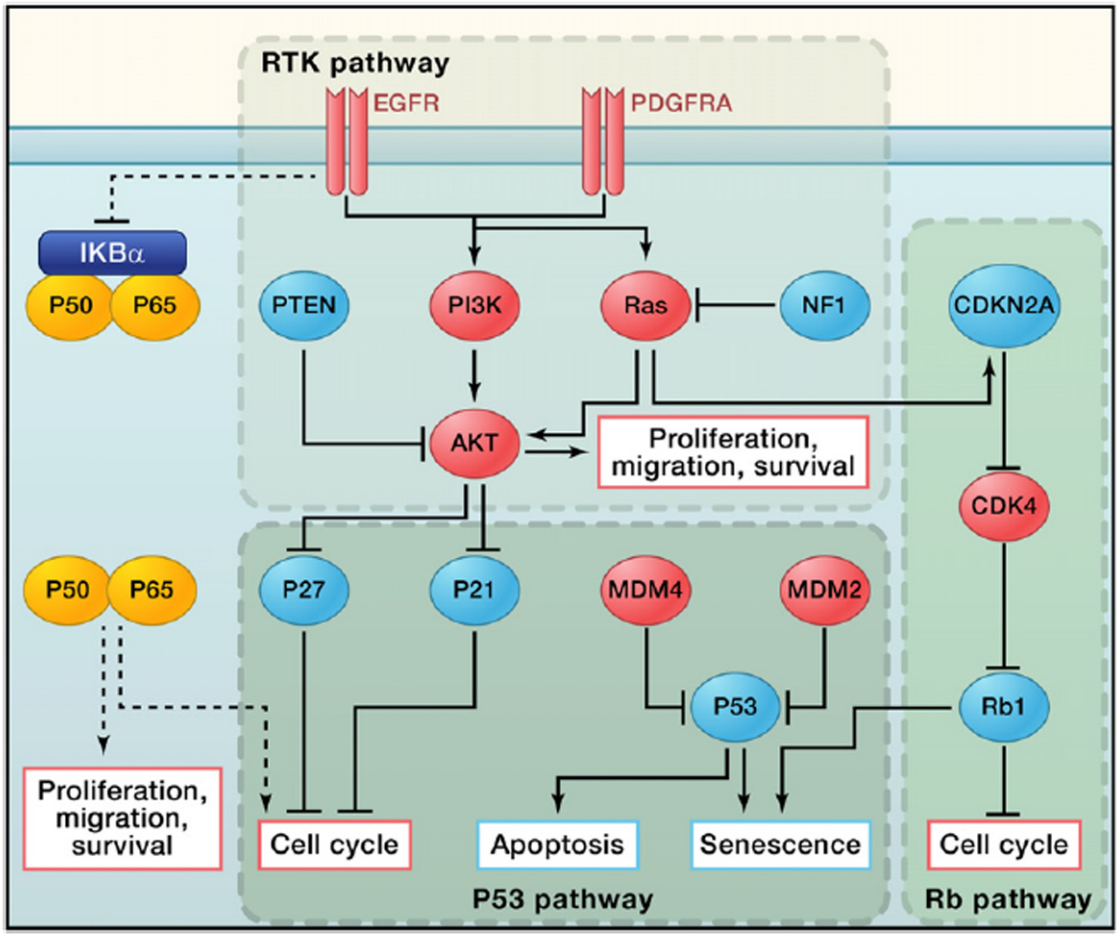


Figure 1.4 . Core Signaling Pathways in Glioma Tumorigenesis. From (45)

Importantly, starting from these data, several Authors tried to molecularly classify GBM into clinically relevant subgroups. For example, Verhaak et al. performed unsupervised clustering analysis of the available GBM data set and grouped the tumors into four subtypes termed proneural, neural, mesenchymal, and classical (46). Interestingly, these subtypes were characterized by specific genetic alterations(46). Moreover, the prognostic value of the molecular sub-classification has also been evaluated, with several studies suggesting that gliomas with expression of genes associated with neurogenesis (proneural subtype) generally correlate with marginally improved survival, while gliomas with mesenchymal gene expression usually have a poorer outcome (1 year for proneural versus 0.6 years for mesenchymal) (47). Moreover, although gliomas with proliferative or mesenchymal characteristics generally have a worse outcome, several studies have confirmed that these subtypes are also more sensitive to combinational radiation and chemotherapy(46).

Therefore, in HGG, genetic studies have contributed to dissect the basis of GBM heterogeneity and to demonstrate that, despite a similar histological appearance, different classes of diseases can be identify on molecular basis. This novel approach would allow a more precise classification system that accurately reflects the cellular, genetic, and molecular basis of gliomagenesis, and that can be a prerequisite for identifying subsets of patients characterized by different prognosis and by a distinctive responsiveness to specific adjuvant therapies. In conclusion, this would ultimately allow achieving individualized clinical care of glioma patients(47). Unfortunately, these studies have not been performed in LGG.

1.2.5 Glioma therapy

The standard therapy for newly diagnosed High Grade Glioma involves radiotherapy, chemotherapy and surgical resection, when feasible. Surgery is commonly used as the initial therapeutic approach, in order to remove the glioma and obtain tissue for diagnosis (35,46). Tumor resection is of prognostic value (47) and thus may be beneficial to attempt maximal tumor resection, provided that neurological function is not compromised by the extent of resection. Advances such as MRI-guided neuronavigation, intraoperative MRI, functional MRI, intraoperative mapping and fluorescence-guided surgery have improved the safety of surgery and increased the extent of resection that can be achieved (46). When microsurgical resection is not feasible (e.g. due to location of the tumour or impaired clinical condition of the patient), a stereotactic serial biopsy should be performed to reach a histological diagnosis, possibly taking into consideration molecular markers (LOH 1p/19q, MGMT promoter methylation) (35). Implantation of chemotherapy impregnated wafers (carmustine polymers) into the resection cavity before radiotherapy, has been shown to marginally improve median survival in patients who undergo radical tumour resection, compared to radiotherapy alone. However, subgroup analysis has not shown significant benefits for patients with glioblastoma **(37)**

1.2.5.1 RADIOTHERAPY AND CHEMOTHERAPY

Standard treatment of glioblastoma currently consists of maximal surgical resection, radiotherapy, and concomitant and adjuvant chemotherapy. Post operative radiation treatment, commonly administered to a total dose of 60 Gy delivered in 2 Gy fractions over 30 days, is routinely performed and significantly improves survival of glioblastoma patients (48). The chemotherapeutic standard of care for patients with newly diagnosed glioblastoma only changed

back in 2005, when the concomitant and adjuvant application of the alkylating agent Temozolomide (TMZ) proved to increase median and 2 year survival of patients when added to radiation therapy (49). TMZ is an orally available prodrug which penetrates into the brain and methylates guanine residues in the DNA, leading to base/base mismatches which result in cell death. Unfortunately, resistance to this drug is mediated by the DNA repair protein O^6methylguanine-DNA-methyltransferase (MGMT), through its ability to remove methyl/alkyl groups from the O^6-position of guanine and thereby preventing TMZ induced DNA damage. Approximately 50% of the patients, namely those with a methylated/epigenetically silenced *MGMT* promoter, i.e. no expression of MGMT, benefit from temozolomide whereas those with an unmethylated *MGMT* promoter do not (50). DNA methylation of MGMT is therefore considered to be a predictive marker of sensitivity to TMZ treatment. Other treatments for glioblastoma either upfront or at recurrence include the use of other alkylating agents (e.g.lomustine or carmustine),procarbazine,topoisomerase inhibitors and at least in several countries, antiangiogenic treatments such as the antibody against vascular endothelial growth factor VEGF ,bevacizumab (51, 52).

1.2.5.2 IMMUNOTHERAPY

The Central Nervous System (CNS) is considered an immune privileged organ (53)-(54). This adaptation of the CNS has been attributed to two morphological peculiarities; i.e. the blood brain barrier, blocking the efferent pathways of the immune system and the absence of classic draining lymph vessels blocking its efferent way. Thus, it is believed that antigens in the CNS are largely ignored by the immune system.

On the other hand, it is believed that the brain is not completely silenced immunologically. In fact, the presence of tumor-infiltrating lymphocytes (TIL) suggest that some anti-tumoral responses are noted (55),(56).

The advantage of generating an immune response against the neoplasia is the power of destroying the residual tumor, which is resistant or in inapproachable areas. Gliomas express tumor-associated antigens (TAA), which can induce the immune response primarily by activating cytotoxic T-lymphocytes (57). Due to this observation the hypothesis of a vaccine has been considered.

Different approaches can be imagined. The first one is to isolate the patient tumoral cells, inactivate them and use them to stimulate the immune system. Unfortunately, this option is time consuming, incompatible with the rapid growth of the GBM.

Secondly, the surgically resected tumor can be incubated with dendritic cells (DC) taken from the patient. The DC act as antigen-presenting cells and, when reinfused, migrate to the cervical lymph nodes and give place to a T-mediated immune response. Implementation of T cell infiltration in the remaining tumor, where a notable part of it are cytotoxic T cells, has been observed in phase I trials that use this strategy (57).

Approaches that involve dendritic cells and peptides for the induction of an anti-tumoral response and evaluation of the overall survival of glioma affected patients, seem promising and free from major side effects.

Further to that however, there are several types of glioma, so it seems necessary to evaluate the genetic-molecular abnormalities on the single patient to design new personalized therapies.

1.2.5.3 GENE THERAPY

Gene therapy involves the transfer of genes inside the tumor using viral, murine retroviral and human adenoviral vectors. Several genes can be introduced, able to activate chemotherapeutic agents, correct defective pathways and stimulate the immune response against the tumor.

There is flourishing literature on gene therapy and the use of oncolytic viruses, such as the use of herpes oncolytic virus, HSV. This therapeutic agent is promising, because it is selective for neoplastic stem cells and it is able to inhibit self-renewal in the non-susceptible subpopulation.

An approach mediated by herpes simplex virus 1 contemplates the transfer of thymidine kinase (TK) gene, which is a virus that activates ganciclovir, converting it into its toxic metabolite. This gene, incorporated within the tumor cells' DNA, can cause death of these cells by apoptosis.

Further studies focus on the use of many interleuchin codifying genes (52).

Another investigation field consists on the analysis of the TNF-related apoptosis-inducing ligand (TRAIL) effects, presumably able to selectively kill the cancer cells. However, reduced clinical efficiency due to short half-life and the resistance of neoplastic cells has been noticed.

Having considered the very high heterogeneity of gliomas, gene therapy in gliomas is limited by the reduced survival of the viral vectors and by their difficulty to get to the neoplastic cells which infiltrate the cerebral parenchyma.

The latest gene therapy involves the construction of mesenchymal stem cell-like vectors, which are supposed to skip the viral vector limits.

1.2.5.4 POTENTIAL USE OF STEM CELLS

Traditional anti-tumoral therapies, such as cytotoxic and radiotherapy, selectively attack the tumoral mass and do not specifically address the cancer stem cells (CSCs).

Targeting the CSCs is the ultimate goal of recent studies, therefore new therapeutic strategies are mostly addressed to understand the signaling pathways involved in stem cell renewal. The aim is to obtain molecules that are not only able to reduce the neoplastic mass, but are also able to eradicate the CSCs, responsible for the recurrence.

In order to create a therapy that is able to selectively target cancer stem cells, it is necessary to characterize these cells and thus understand how they differ from non-staminal or normal cells.

For example, CD133+ cancer cells secrete more VEGF than the normal counterpart. It has also been demonstrated that antiVEGF therapies, such as bevacizumab, (which is a humanized monoclonal antibody which recognizes VEGF-A), neutralize proangiogenic effects and reduce the peri-lesional edema, further reducing the desametasone doses required (58).

Infact, recently it has been proposed that stem cells can be used as novel therapy, for example

MSCs can be utilized to deliver prodrug-converting enzyme. A pioneer example is the combination of herpes simplex virus-thymidine kinase (HSV-tk) gene engineered MSCs and systemic administration of ganciclovir (59). Within tumors HSV-tk is released by engineered MSCs and converts (phosphorylates) the prodrug ganciclovir into its toxic form, thereby inhibiting DNA synthesis and leading to cell death. This therapeutic regimen has been successfully employed in glioma (60) and pancreatic cancer (61) experimental models. MSCs have been used to deliver another prodrug-converting enzyme, cytosine deaminase. Following sistemic administration, the prodrug 5-fluorocytisine is converted into the highly toxic active drug 5-fluorouracil in tumors. This system has shown therapeutic effectiveness in animal cancer models, such as melanoma (62), colon carcinoma (63), and prostate cancer (64).

It has been shown that the therapeutic effect of MSCs depends on the proportion of the cells in the tumor (65), and it was suggested that several injections of MSCs might be required to achieve a prolonged therapeutic effect (66). Therefore, the approach to increase the retention or homing of MSC in the tumors is warranted. Moreover, the modification of MSCs with antibodies might become a new strategy to deliver these therapeutic molecules which poorly penetrate the blood brain barrier. The combination of antibody-directed targeting to a specific cell population and the natural tropism of MSCs to tumor holds the promise in that respect. Recently, it has been

demonstrated (67)the feasibility of genetic modification of hMSCs to express scFv EGFRvIII (hMSC-scFvEGFRvIII) on the cell surface in order to enhance their targeting to EGFRvIII expressing tumors (67). It has also been demonstrated (67)whether hMSC-scFvEGFRvIII possess the enhanced properties to achieve higher retention in EGFRvIII expressing glial tumor. Most importantly, it has been evaluated the therapeutic response, in particular the growth of U87-EGFRvIII glioma cell in vitro and in vivo, upon exposure to hMSC-scFvEGFRvIII.

1.3 Stem cells and Cancer Stem cells

The stem cell theory of cancer postulated that cancer tissues are characterized by the same hierarchical order that regulate homeostasis in normal tissues(68). Therefore, as in normal tissues a rare population of stem cells is responsible for the turnover and the regenerative response of the organ in which they are endowed, cancer tissues rely on a rare population of cells characterized by stem cell properties(68). Therefore, it has been considered fundamental the comprehension of the properties characterizing stem cells and cancer stem cells and the identification of differences possibly allowing distinguishing (targeting) normal stem cells from cancer stem cells.
More recently, it has been demonstrated that in cancer tissues, besides tumor-initiating cells, other non-tumorigenic stem cells are present and they seem to play a fundamental role in the tumor growth. Specifically, mesenchymal stem cells (MSC) seem to possess a particular tropism for tumor lesions and their presence it has been demonstrated in several tumors, including gliomas. Therefore, even normal stem cells seem to play a role in cancer.

1.3.1 Stem cells

A stem cell is a cell that can continuously produce unaltered daughters and also has the ability to produce daughter cells that have different, more restricted properties (69).
To define a cell as a stem cell, researchers have used four criteria. First, stem cells undergo multiple, sequential long-term self-renewing cell divisions, a prerequisite for sustaining the population, making identical copies of themselves for prolonged periods of time (even for the entire lifetime of an organism). Second, single stem cell-derived daughter cells differentiate into one or more cell types; they can give rise to mature cell types that have characteristic morphological properties and specialized functions (Figure 1.5).

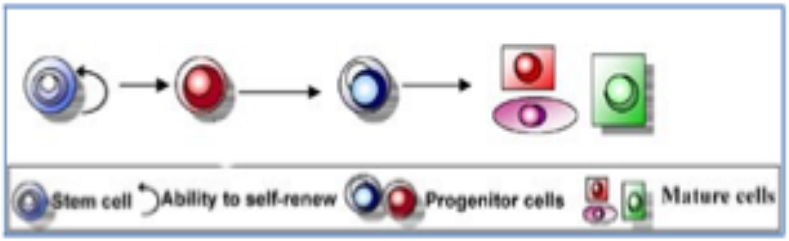


Figure 1. 5. A stem cell is an unspecialized cell that is capable of replicating or self renewing itself and developing into specialized cells of a variety of cell types. The product of a stem cell undergoing division is at least one additional stem cell that has the same capabilities of the originating cell. From (1).

Typically, stem cells generate an intermediate cell type or types before they achieve their fully differentiated state. The intermediate cell is called a precursor or progenitor cell. Progenitor or precursor cells in fetal or adult tissues are partly differentiated cells that divide and give rise to differentiated mature cells (Figure 1.5). Such cells are usually regarded as "committed" to differentiating along a particular cellular development pathway, although this characteristic may not be as definitive as once thought. A third criterion is that stem cells functionally repopulate the tissue of origin when transplanted in a damaged recipient. A final criterion is that stem cells contribute differentiated progeny *in vivo* even in the absence of tissue damage, for the normal turnover of the tissue in which they are located (70).

1.3.1.1 Stem cell plasticity

One of the key feature of stem cells is ***plasticity***, or potency, that is the ability of a stem cell to differentiate into multiple derivatives of one or more germ layers (Figure 1.6).

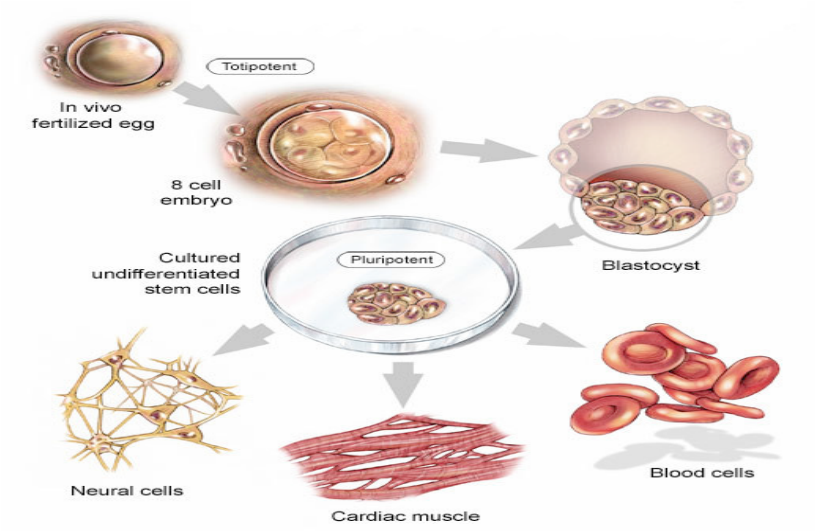


Figure 1.6 . Stem cell potency (www.stemcellresearchfoundation.or

Considering plasticity, at the top of the stem cell tree (the most primitive) is the fertilized oocyte (the zygote) and the descendants of the first divisions. These cells are **totipotent**, able to form the embryo and the trophoblasts of the placenta. The progeny of embryonal stem cells are the precursors for all of the cells of the adult organs. After about 4 days, these totipotent cells begin to specialize, forming a hollow ball of cells, the blastocyst, and a cluster of cells called the inner cell mass (ICM) from which the embryo develops. The ICM cells are considered to be **pluripotent**, able to differentiate into almost all cells that arise from the three germ layers, but not the embryo because they are unable to give rise to the placenta and supporting tissues (Figure 1.6).

During this specialization process, the progeny of the embryonic stem cells loses potential and gain differentiated properties, in a poorly understood process called determination. It is generally accepted that adult tissues contain tissue-determined stem cells responsible for normal tissue renewal. In the adult, the cells responsible for tissue renewal are no longer totipotent, but become more restricted in their ability to form different tissues. An exception to this rule may be germinal cells; in the adult they produce eggs and sperm and are responsible for reproduction. Some tumors of germinal cells (embryonal carcinomas) are able to produce differentiated cell types of all adult organs, as well as placental tissues (71).

Somatic stem or progenitor cells are considered more limited in their potential, and they produce cells that differentiate into mature functioning cells and that are responsible for normal tissue renewal. Most tissues have **multipotent** stem cells, cells capable of producing a limited range of differentiated cell lineages appropriate to their location; for example, small intestinal stem cells can produce all four indigenous lineages (Paneth, goblet, absorptive columnar, and enteroendocrine), while central nervous system (CNS) stem cells have tri-

lineage potential capable of generating neurons, oligodendrocytes, and astrocytes. The bone marrow hematopoietic stem cells (HSCs) are also multipotent, and can normally produce red blood cells, platelets, polymorphonuclear leukocytes, monocytes and lymphocyte precursors. Most, but not all, of these become terminally differentiated in their fully mature forms. The stromal cells of the bone marrow are also multipotent and can produce progeny that become osteoblasts, osteoclasts, chrondrocytes, fibroblasts, or muscle cells. At the bottom of the tree are **unipotent** stem cells, cells capable of generating one specific cell type. Into this category there are epidermal stem cells in the basal layer that produce only keratinized squames and certain adult hepatocytes that have long-term repopulating ability. These adult stem cells in non-germinal tissue are very few in number (Figure 1.7).

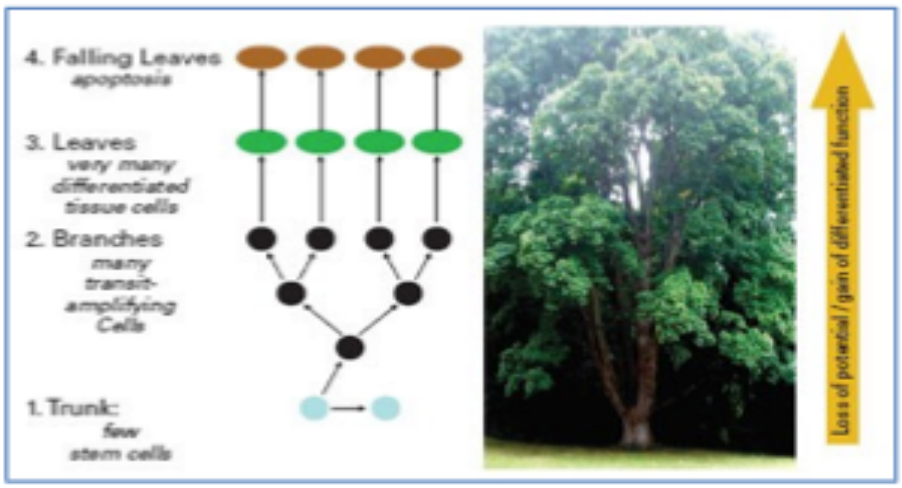


Figure 1. 7 Stem cell lineages in adult tissues and normal tissue renewal. From (3)

Classically, the term transdifferentiation was used for the process of reprogramming cells that have already embarked on a differentiation pathway, and has been used interchangeably with metaplasia and transdetermination. However, it is now appreciated that adult progenitor cells of one tissue may be able to differentiate into mature cells of another tissue type. For example, the adult bone marrow contains small numbers of undifferentiated stem cells having sufficient plasticity to give rise to progeny that can become at least nine different mature cell types, depending to the environment in which they are seeded a concept originally proposed by Cohnheim in 1867.

1.3.1.2 Hierarchical organization of Stem Cells

The first tissue in which SC have been identified is the bone marrow. For this reason Hematopoietic Stem Cells (HSC) are the most characterized stem cells, and their hierarchical organization is considered a reference model for the SC of all the other tissues (Figure 1.8) (72).

Normal tissue renewal is provided by a rare population of tissue stem cells that divide asymmetrically to give rise to one daughter cell that retains SC properties and one daughter cell that begins the process of determination. In most organs, the normal replacement of terminally differentiated cells is accomplished by proliferation of progenitor cells or transit-amplifying cells. Transit-amplifying cells provide an expanded population of proliferating tissue determined cells and produce progeny that differentiate into more mature cells that can no longer proliferate and eventually die.

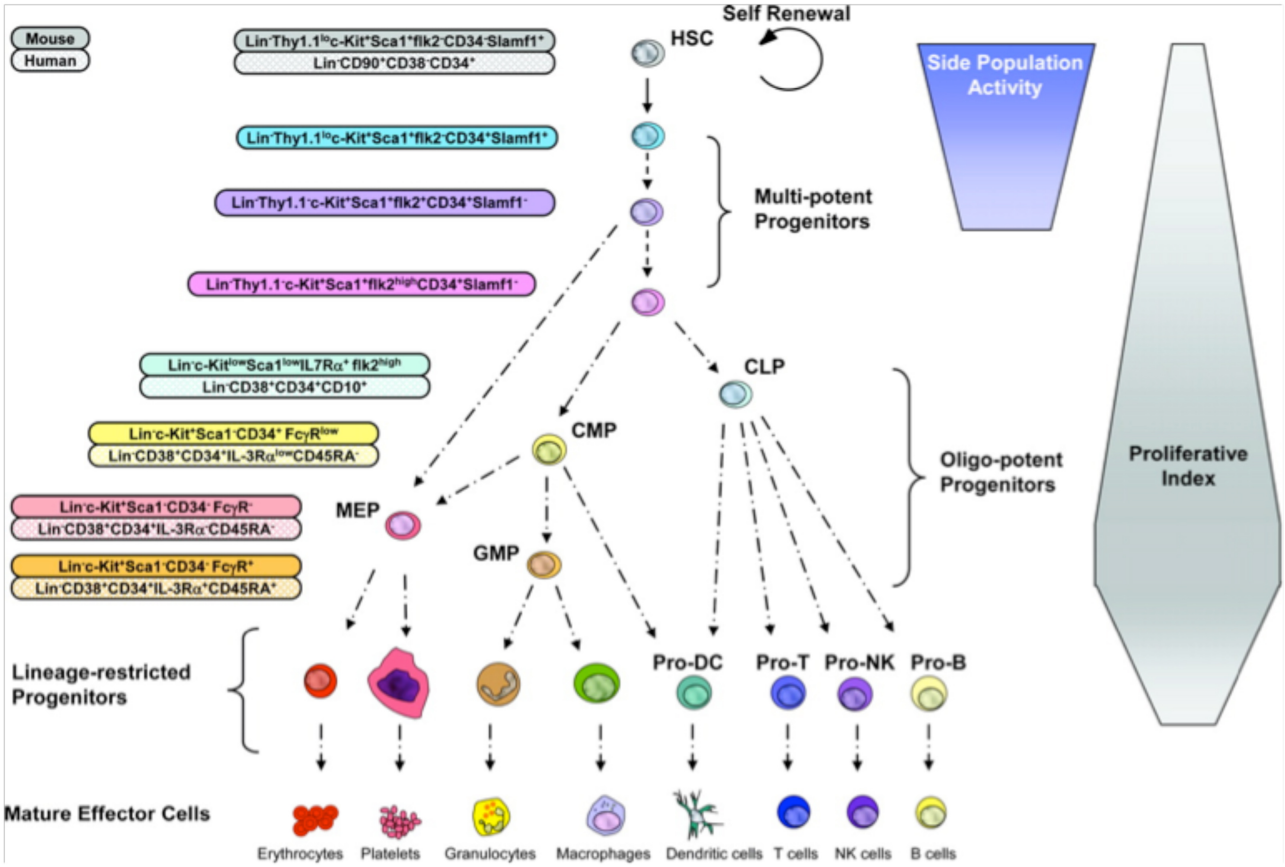


Figure 1. 8. Hierarchical Organization of hematopoiesis. From (72).

1.3.1.3 Molecular markers of stemness

Besides the properties that were previously described, adult stem cells express specific markers. These molecular markers are very useful for the identification/isolation of the stem cells in the tissue. Although the markers characterizing in vivo HSC are well known, localization and characterization of stem cells of all the other tissue is frequently still under investigation (73).

Among the molecular markers of stemness, a key role is played by ***pluripotent-state specific transcription factors***, such as Nanog and Oct3/4 (74). Genetic studies have demonstrated that Oct 3/4 and Nanog are essential regulators for the development and for the identification of embryonic stem cells (75). The loss of Nanog and Oct3/4 usually determines the lack of pluripotency (75). Recently many studies suggest that that Nanog could have a role in the generation of the pluripotent state of embryonic stem cells, but not in its maintenance.

Sox-2 represents another important pluripotent-state specific transcription factor, which is expressed in pluripotent embryonic cells and germ cells. Together with Oct-3/4 and Nanog, it is essential for the preservation of the self-renewal properties(75).

According to different authors, Nanog, Sox-2 and Oct3/4 cooperate within a regulatory circuitry (autoregolatory loop) in which, not only they regulate their own promoters, but they can bind promoters of other genes involved in the maintenance of pluripotency, activating them, and they can also bind promoters of genes related to cell differentiation, suppressing them (Figure 1.9) (2). Most of the transcriptionally silent development regulators tergeted by Nanog, Sox-2 and Oct3/4 are also occupied by Polycomb Group (PcG) proteins (76, 77) which are epigenetic regulators that facilitate maintenance of cell state through gene silencing (Figure 1.9) (2).

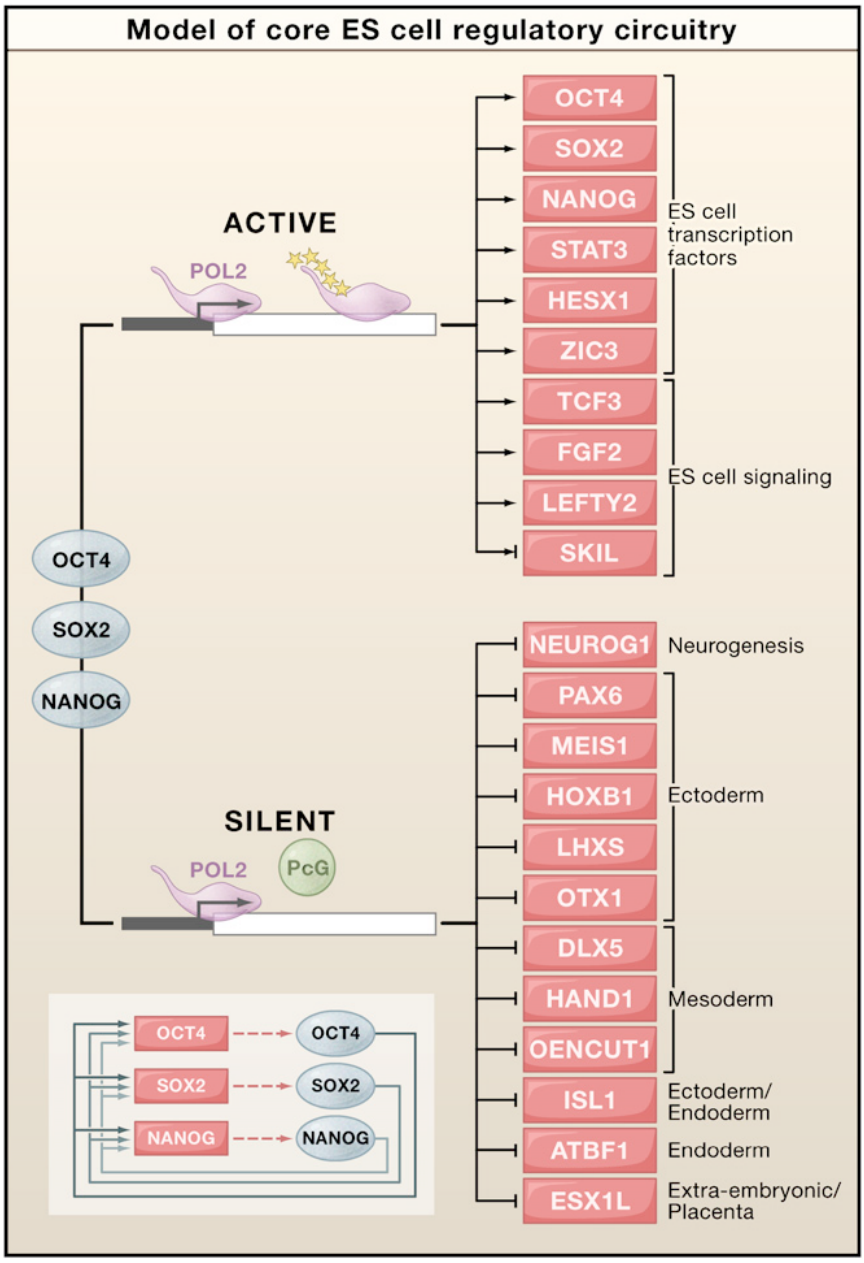


Figure 1. 9. Model of Core ES Cell Regulatory Circuity. From (2)

The Oct4, Sox2, and Nanog transcription factors (blue) occupy actively transcribed genes, including transcription factors and signaling components necessary to maintain the ES cell state. The three regulators also occupy silent genes encoding transcription factors that, if expressed, would promote other more differentiated cell states. At this latter set of genes, RNA polymerase II (POL2) initiates transcription but does not produce complete transcripts due to the repressive action of PcG proteins.

The PcG proteins prevent RNA polymerase from transitioning into a fully modified transcription elongation apparatus (represented by phosphorylated ‘‘stars’’ on the tail of the POL2 enzyme).
The interconnected autoregulatory loop, where Oct4, Nanog, and Sox2 bind together at each of their own promoters, is shown (bottom left).

Other well-recognized stem cell markers are:

- CD34: is a transmembrane protein that is strongly expressed on hematopoietic stem/progenitor cells (HSCs) (78).
- CD133: is a transmembrane protein, initially described as a marker of hematopoietic stem cells, but subsequently identified also in embryonic and neural stem cells (79)
- c-kit or CD117: is a tyrosine kinase receptor that, interacting with its ligant, named Stem Cell Facror (SCF), play an important role in hematopoietic stem cells, cardiac stem cells and germ cells (80).
- Musashi: is a group of RNA-binding proteins characterized by two RNA recognition motifs (RRMs) and is evolutionarily conserved. In mammals, two isoforms of this family, Msi1 and Msi2, are co-expressed in neural precursor cells, including Neural Stem Cells (NSCs) (81).
- Nestin: a protein *marker* for *neural stem cells.*

These stem cell markers have been used to identify stem cells within the tissue, and in the case of surface markers, such as CD34, CD133 and CD117, they have been used to isolate stem cells from the tissues.

1.3.1.4 Human Mesenchymal Stem Cells

Mesenchymal Stem Cells (MSC) were initially identified within the adult bone marrow (BM), where they are responsible for the generation of the BM microenvironment (82, 83). Nowadays, MSC have been identified, in most of the tissues, as a multipotent progenitor cell pool with the ability to differentiate into multiple mesoderm lineages, both in the course of normal tissue homeostasis and during injury(84). In fact, MSC act as trophic mediators during tissue repair, generating bioactive molecules that help in tissue regeneration following injury(84).
Importantly, MSC seem to play a comparable role in cases of malignancy and are becoming increasingly appreciated as critical components of the tumor microenvironment(84). MSC home to developing tumors with great affinity, where they can: 1.differentiate into the different cell types composing the tumor microenvironment (including the tumor associated fibroblast-TAF- see below); 2. increase cancer cell proliferation, motility, invasion and metastasis; 3. promote

angiogenesis and 4. suppress anti-tumor immune responses(84, 85). These multifaceted roles emerge as a product of reciprocal interactions occurring between MSCs and cancer cells and serve to alter the tumor milieu, favoring a dynamic co-evolution of both tumor and stromal tissues that favors tumor progression(84).

This emerging role of MSC in cancer, it has opened a way to numerous studies aimed at considering MSC as a putative novel therapeutic target or, taking advantage of MSC tropism for cancer, even as a novel therapeutic tool. In fact, gene modified MSC were successfully utilized for the treatment of a murine model of GBM(86).

1.3.2 Cancer stem cells

In the last years, the discrepancy form the classical view, in which neoplasia are formed by a homogeneous population of cells, stimulated the studies about cancer stem cells (CSC). For example, clinical and experimental observations demonstrated a frequent failure in inducing a tumor in animal model injected with the bulk tumor cells(68).

Therefore, Authors started to hypothesized that just a small fraction of the cells composing the neoplasia possess the ability to self-renew and to generate all the other differentiated components of the tumor (Figure 1.10).

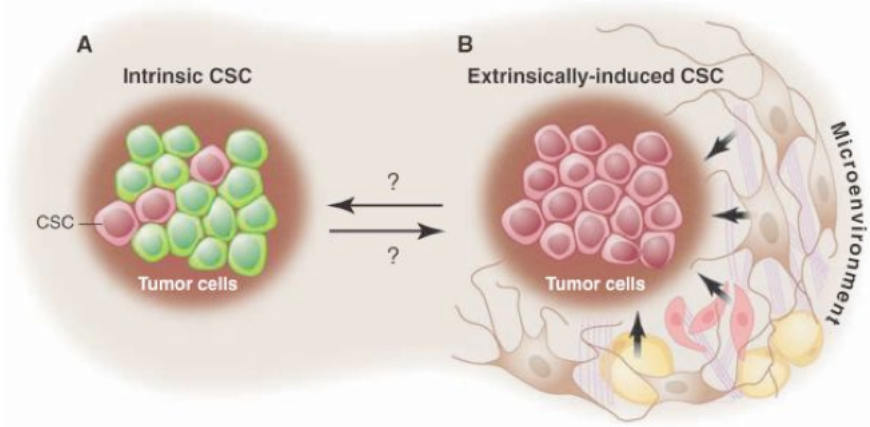


Figure 1. 10. CSC models. (A) The intrinsic model suggests that specific subpopulations within a tumor (pink cells) possess the functional properties of CSCs. (B) The extrinsic model proposes that all tumor cells are functionally equivalent and display heterogeneous behaviors as a function of extrinsic (microenviron-mental) cues. From (87).

In 1997 Dick et al. first described the existence of cancer stem cells, named tumor-initiating cells (TIC), in human acute myeloid leukemia (88). Authors demonstrated that a subpopulation of cells that were CD34+ CD38-, were able to self renewal and to generate, when injected in NOD-SCID mice (non-obese e diabetic-severe and combined immunodeficient systems), a myeloid acute leukemia phenotypically identical to the starting tumor. The phenotype of TIC was very similar to the one described for normal hematopoietic stem cells suggesting that tumor and normal tissues are governed by a similar hierarchic model. One of the most important consequences of this hierarchic model is that cells inside the tumor are not all the same in terms of self-renewal, proliferating and differentiation capacity, and only the very primitive cells are characterized by the attitude to start and maintain the tumor and can be responsible of recurrences and metastasis(89).

This human leukemia model represented the paradigm for the subsequent studies focused on solid tumors, such as mammary carcinoma (1) glioma (79, 90), melanoma, thyroid cancer, lung cancer and gastroenteric tumors (91, 92).

Solid tumors resulted to be the more complex as they contain not only tumor cells, but also stromal cells, which interactions can change the first one's properties. Moreover, CSC share several stem-cell related properties with non neoplastic stem cells, especially the ones related to self-renewal (93).

In fact, pathways related to Wnt, Sonic Hedgehog and Notch, which normally regulate the normal cells self-renewal, are present and up-regulated in several cancers (Figure 1.11) (4).

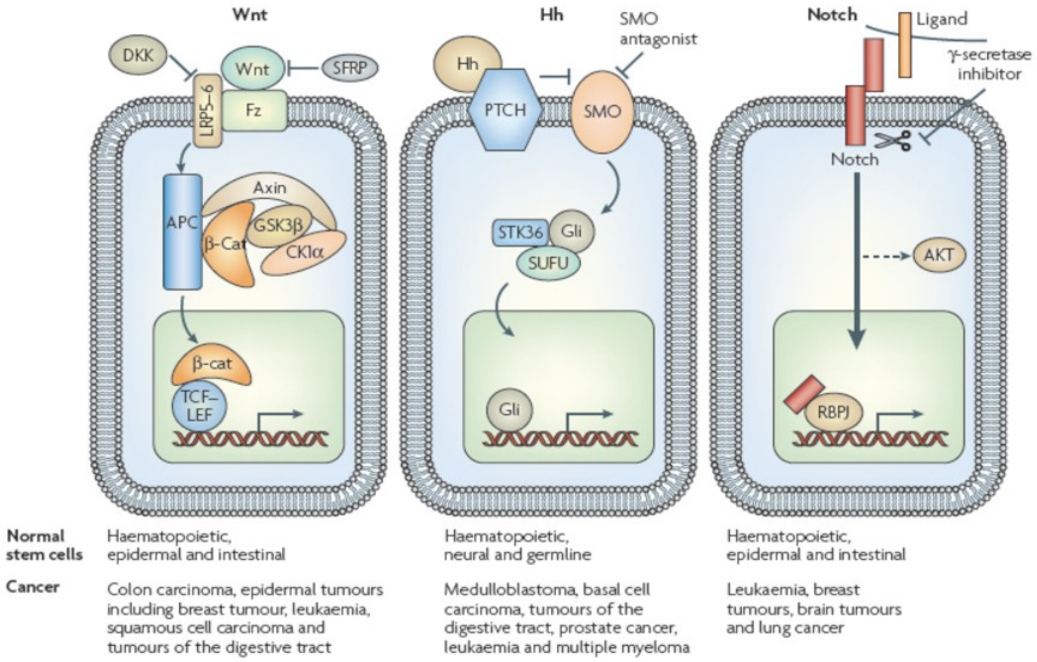


Figure 1.11. Pathway that regulate the self-renewall in normal stem cells and tumor transformationn. From (4).

Importantly, stem cells, whether they are normal or neoplastic, are usually characterized by a major resistance to common therapies due to the up-regulation of drug-resistance pathways(68, 94).

Regarding the origin, there are two hypotheses on the origin of the cancer stem cells (68):

- CSC may result from a normal stem cell that undergoes several successive mutations and acquires a neoplastic phenotype (Figure 1.12);
- CSC may originate from a progenitor cells that, by mutation, re-acquire self-renewal properties.

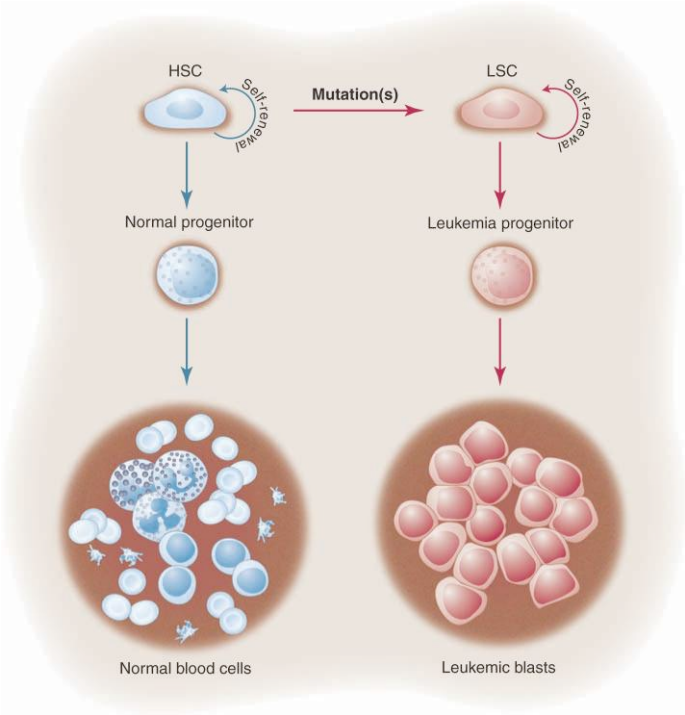


Figure 1.12. Initial studies in leukemia provided the paradigm for the general CSC model. As shown on the left side of the figure, a hematopoietic stem cell (HSC) gives rise to normal progenitors and mature blood cells. The original model suggests that the HSC undergoes mutation(s) that give rise to its malignant counterpart, the leukemia stem cell (LSC). The LSC retains some degree of developmental potential, generating the leukemia progenitor and leukemic blast cells, which differ in their biological properties from the parent LSC. As in normal hematopoiesis, the stem cell maintains the ability to undergo self-renewal and thereby perpetuate the leukemia population. From (87).

There are experimental evidences, *in vivo* and *in vitro* in favor of both possibilities (94-96).

The concept that a specific subpopulation of tumor cells possesses stem cell properties implies that only CSCs are mostly responsible for the tumor biology and development.

Recently, the stem cell theory for cancer has been questioned. Specifically, Quintana's group showed that modified xenotransplantation assay conditions, including the use of more highly immunocompromised NOD/SCID interleukin-2 receptor gamma chain null (Il2rg(-/-)) mice, could highly increase the detection of tumorigenic melanoma cells, suggesting that, at least in some cancers, tumorigenic cells are quite frequent(97).

One of the possible explanations for these results is the fact that the cancer microenvironment (stromal fibroblasts, adipocytes, and endothelial cells, as well as the extracellular matrix) and the immune system play important roles in cancer progression (98, 99). Consequently, the xenograft model could not offer an appropriate microenvironment for the growth of human tumors, because of the differences between the mouse and human and the lack of an intact immune system. These limitations can be important when evaluating the tumor-initiating capacity of these human cancer cells. Thus, it is possible that the subpopulation of cells that appeared non-tumorigenic in a NOD-Scid mouse model might actually be tumorigenic in the presence of the appropriate

microenviroment. In other words, tumor cells might be functionally homogeneous, with heterogeneous potential arising as a consequence of extrinsic cues or of their lack.
It is not clear yet, if the different types of neoplastic stem cells preserved same characteristics as it was showed in stem cells. Another important aspect is to understand how cancer cells are able to resistant to common therapies and treatment. Infine, it would be clinically useful to assess the survival based on the number of CSC and not on the residual tumor mass (94).

In conclusion, the CSC paradigm refers to the ability of a subpopulation of cancer cells to initiate tumorigenesis by undergoing self-renewal and differentiation, like normal stem cells, whereas the remaining majority of the cells are more "differentiated" and lack of these properties. This concept, although debated, has opened new horizons for understanding the biology of cancer and for identifying new therapeutic modalities apt to eliminate the cells responsible for tumor self-renewal.

1.3.3 Glioma-initiating cells

Vescovi gave a functional definition of brain tumor stem cells. Specifically, brain tumor cells could be considered as stem cells if they show: 1. Cancer-initiating ability upon orthotopic implantation; 2. Extensive self-renewal capacity either ex vivo or in vivo; 3. Karyotypic or genetic alterations; 4. Aberrant differentiation properties; 5. Capacity to generate non-tumorigenic end cells, and 6. multilineage differentiation capacity(100). Because this subpopulation of glioma cells, generally called glioma stem cells (GSC), may play an extremely critical role in the initiation and recurrence of gliomas, plenty of studies focusing on GSC have been published.
Nowadays, two methods have been applied to isolate GSC, either based on the prospective isolation of specific cell sub-population, or on selective growth conditions.
Regarding the ***first method***, Glioma Stem Cells (GSC) were among the first cells to be defined as a small population of cells expressing the cell surface marker CD133 (Figure 1.13)(79). This latter, formerly known as PROML-1 or AC133, was originally discovered as the equivalent to mouse prominin, a pentaspan transmembrane glycoprotein of murine neuroepithelial stem cells located in plasma membrane protrusions(101, 102). Although no interacting proteins are known, a role in cell polarity and cell migration was suggested due to its specific localization(103). Whereas CD133 is expressed in a variety of human tissues, the CD133 antigen with the glycosylated epitope AC133 is mainly restricted to stem cells(101, 102). When isolated from human brain tumors, CD133+ displays stem cell properties in vitro, such as enhanced capacity for proliferation, self-renewal, differentiation and neurosphere-like growth(79). More importantly, CD133+, but not CD133-,

tumor cells were able to reconstitute, when injected in vivo into immune-deficient nude mice, a tumor characterized by the same phenotype of the patient's original tumor (79, 90). In fact, injection of as few as 100 CD133+ cells produced a tumor that could be serially transplanted, whereas injection of 10^5 CD133– cells failed to produce any tumor(104). Importantly, the small fraction of CD133+ cells seemed to be preferentially resistant to chemotherapeutic agents and radiation, and expressed higher levels of mRNA for the ABC-transporter BCRP1, the O6–methylguanine–DNAmethyltransferase, markers associated with neural precursors, and negative regulators of apoptosis, and could thus be responsible for post-treatment recurrence(105, 106).

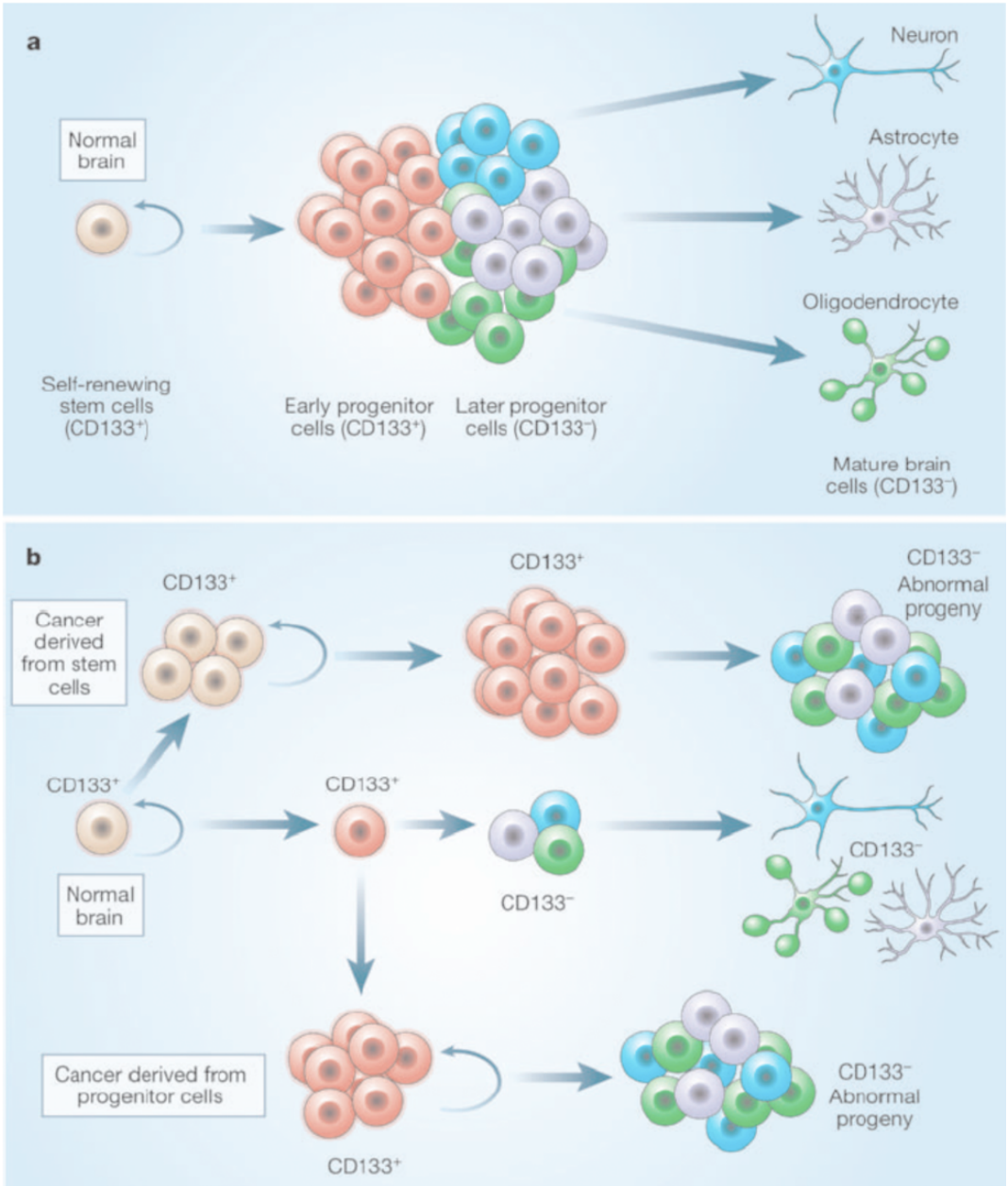


Figure 1.13 . CD133 as a marker of brain stem cells and glioma-tumor initiating cells. (34).

Although the model of Singh demonstrated that $CD133^+$ cells, could initiate the tumors, because endowed with stem cells properties, it's not clear yet, if the $CD133^+$ tumor cells, derive from normal progenitors or from normal progenitors that reacquire characteristics of stemness (Figure 1.13). The frequency of Cancer Stem Cells in brain tumors is higher when they compared to the other cancers, this can be justified by the fact that they are the most aggressive forms of human cancer (79).

However, recent reports indicate that this initially proposed model may represent an over-simplification and stem cell-specificity of the epitope detected by the antibody AC133 (i.e. glycosylated prominin, CD133)(107) has been questioned(102). GBM cells may acquire CD133 after xenotransplanation(108), conversely CD133+ and CD133- cells may have similar tumorigenic potential(109, 110). In addition, CD133 does not appear to be essential for stem cell-like properties, as testified by the identification of subgroups of GBM derived by CD133- GSC(111-113). Thus, stem cell-specific markers other than CD133, e. g. CD15/SSEA-1 and integrin a6, have been described, but there is not yet consensus on the optimal markers for GSC in GBM(113-116).

The ***second method*** utilized to isolate glioma stem cells relies on the ability of these tumor-initiating cells to grow as neurospheres. This assay was originally optimized for the isolation of normal stem cells from healthy brain tissue (117).

Neurosphere assays are carried out culturing glioma-derived cells on non-adherent plates and in selective serum-free media usually added with mitogens including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) or both(102). This culture condition would favor the survival and expansion of stem-like cells responsive to these cytokines and able to continually divide and form multipotent clonal spheres called neurospheres, while the more differentiated cells, incapable of self-renewal and multipotency, are lost with serial passages(102).

Neurospheres were initially isolated from both adult(118) and pediatric tumors(119, 120). Authors demonstrated that brain tumor cells were able to produce proliferating neurospheres that could be passaged at clonal density and differentiated into cells of both neuronal and glial lineage. However, with respect to neural stem cells, tumor neurospheres were characterized by an impaired differentiation capacity mainly favoring the differentiation along the phenotype of the tumor of origin(119, 120) or characterized by generation of cells co-expressing glial and neuronal markers(118-120). Importantly, neurospheres expressed many genes characteristic of NSC-derived spheres, such as CD133(119, 120).

Subsequently, Galli et al demonstrated that GBM-derived neurospheres were characterized by genetic aberration and were able, once injected both subcutaneously and orthotopically into immunocompromised animal, to generate tumor xenograft histologically resembling the original tumor(90). Importantly, tumor xenograft could be serially transplanted thus confirming the in vivo

self-renewal and tumorigenic capacity of neurospheres(90). Many papers have subsequently confirmed the results obtained by Galli(102) and the superiority of the serum-free culture-method above the standard serum-supplemented culture conditions(121).
Most importantly, in vivo studies have shown that neurosphere formation is a significant predictor of clinical outcome in glioma patients, independent of Ki67 proliferation index, and is a robust, independent predictor of glioma tumor progression(122).
Unfortunately, both methods present some drawbacks, making not feasible, to date, to apply high-throughput in vitro analyses to study GSC biology and to search for compounds that selectively kill cancer stem cells without killing the normal cells of the CNS. In fact, freshly isolated CD133 positive cells represent a small, although variable, fraction of the tumor cells and the efficiency of neurosphere assays for producing GSC lines is considered rather low(102). Moreover, neurospheres can be effectively obtained only from 30-50% of GBM, but not from adult low-grade gliomas(90, 120).

In conclusion, it is possible to isolate glioma-initiating stem cells both prospectively, utilizing specific surface markers, or taking advantage of the ability of this cells to grow as neurosphers. Unfortunately, these methods are mostly effective in GBM and isolated/obtained cells cannot always be analyzed by high-throughput methods thus making more difficult their study.

1.4 The Tumor microenvironment

Since 2000, Hanahan and Weinberg have postulated that tumors are not simply constituted by proliferating cancer cells(123) but they resulted composed of multiple distinct cell types involved in heterotypic interactions with one another(124). Importantly, this *tumor-associated stroma*, although constituted by recruited normal cells, is not a passive spectator but it plays an active role in tumorigenesis; as such, these stromal cells contribute to the development and expression of certain hallmark properties(124), such as sustaining tumor proliferation, inducing angiogenesis, avoiding immune destruction, deregulating cellular energetics, inducing invasion and metastasis(125-127).

Importantly, many Authors pointed to a possible prognostic as well as predictive function of the stromal elements of tumors, and novel targeting opportunities within the tumor microenvironment are under investigation(128-130).

Specifically, the tumor microenvironment is considered to play pro-inflammatory, pro-angiogenic and pro-mutagenic roles.

The role of ***chronic inflammation*** in cancer is well known; paradigmatic is the development of gastric lymphoma in patients with chronic gastritis caused by Helicobacter Pylori, or of cancer colon in patients with inflammatory bowel disease. The cells of the immune system release soluble growth factors, enzymes that play an important role in the remodeling of the matrix, reactive oxygen radicals and other molecules have effected on the cell proliferation, on the angiogenesis and in metastasis. At the same time, tumor cells release inflammatory cytokines that can recall lymphocytes, monocytes-macrophages and neutrophils. Accordingly, the chemotactic factor CCL2 is associated with a worse prognosis in the cancers of breast, bladder and cervix (131) and Colony Stimulating Factor 1 (CSF1) over-expressed in some cancers, while CSF-1 null mice do not exhibit accumulation of macrophages in the vicinity of the tumor and are characterized by a slow progression and inability to metastasize (132). The tumor microenvironment contributes to ***angiogenesis*** since it is characterized by and increased secretion of the Matrix Metalloproteasis 9 (MMP3), and Matrix Metalloproteasis 13 (MMP13), both of them responsible for the remodeling of the extracellular matrix. Several cells present in the microenvironment, e.g. tumor associated fibroblasts and macrophages, can produce these factors that are responsible for the angiogenic switch' necessary for the metastatic spread and the growth and the possible metastatic spread of cancer cells (133). Finally, macrophages, mast cells and other white blood cells also release other pro-angiogenic factors such as VEGF, angiopoietin-1, bFGF, TGF-b, PDGF, TNF-alpha (134), (104). The extracellular matrix and the basement membrane components such as fibronectin and collagen type IV, provide both pro-angiogenic and anti-angiogenic signals depending on their structural integrity and assembly process. Fibronectin and collagen type IV show a pro-angiogenic role due to their ability to establish links with integrins. The over-expression of thrombospondin-1 (TSP1) in squamous cell skin tumors is another component of the baseline membrane, that prevents the penetration of vessels by delaying the invasion. It is believed that the antiangiogenetic role of TSP1 is mediated by integrins(135).

Finally, the microenvironment seems to play a ***mutagenic role***: one probable mechanism is the production of reactive oxygen radicals that induce, reduction of the pH, which leads to local mutagenic effects. Moinfar and Collaborates, have reported several genetic alterations and LOH (loss of heterozygosity) in stromal cells, adjacent to the primary breast tumor (136).

1.4.1 Tumor associated fibroblasts

Nowadays the tumor microenvironment has been studied mainly in solid tumor of epithelial origin; specifically Authors focused their attention on the so-called cancer associated fibroblast (CAF) or tumor associated fibroblasts (TAF) that represent the most abundant cell types in the tumor stroma(128, 137, 138).

Five aspects can define TAF (Figure 1.14):

1. Expression of markers such as Fibroblast Activating Protein (FAP), which is selectively expressed on TAF, and Fibroblasts Specific Protein (FSP), that is normally expressed by MSC.
2. Expression of proteins involved in the processes of invasion and remodeling of surrounding stroma such Matrix metalloproteinases (MMP), stromelysin-1 (SL-1) thrombospondin-1 (TSP-1) and tenascin-C (TN-C);
3. Expression of proteins associated with neovascularization such as Muscle Actin Smooth (SMA) (85, 139-141), desmin, Stromal-cell Derived Factor-1 (SDF-1/CXCL-12) and Vascular Endothelial Growth Factor (VEGF);
4. Production of growth factors such as basic Fibroblast Growth Factor basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), interleukin-6 (IL-6), and hepatocyte growth factor (HGF), many of which can also be derived from carcinoma cells.
5. Secretion of chemokines (MCP1, Monocyte Chemotactic Protein 1) (142).

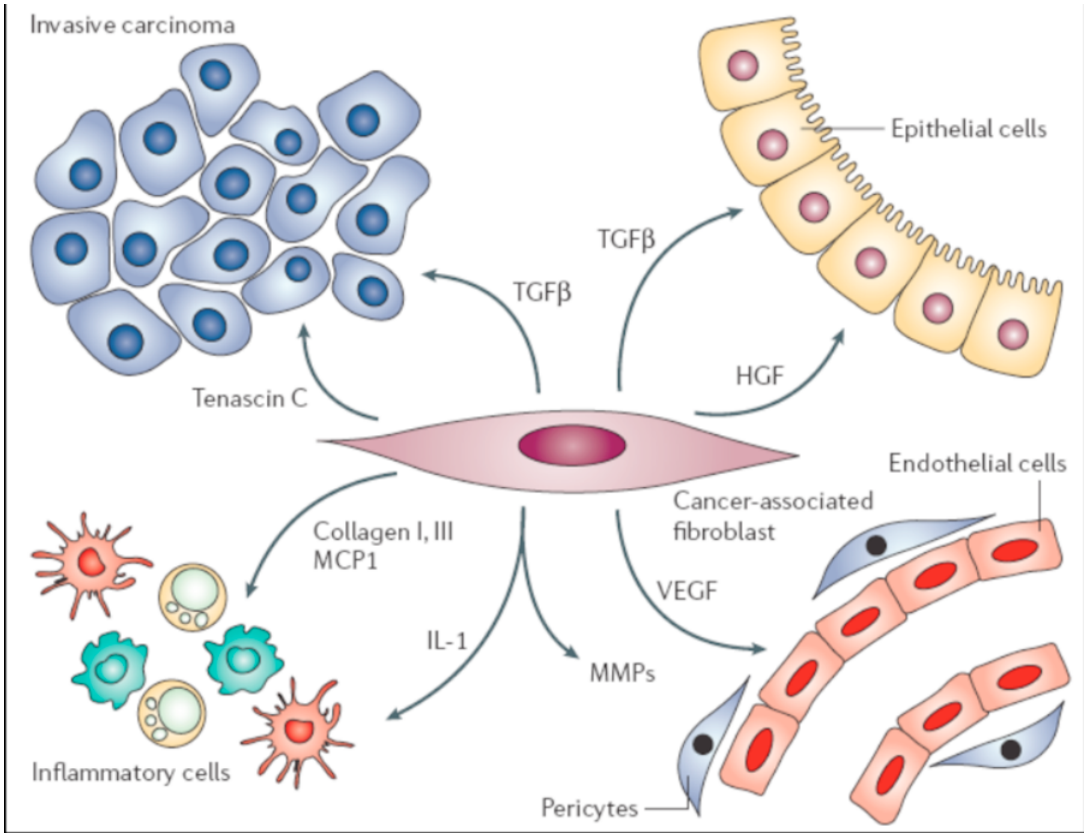


Figure 1. 14. Factors produced by TAF and their target within the tumor microenvironment. From (143).

Although the ***biological origin*** of CAF is still undetermined, data in the literature currently support four possible origins(139):

- the recruitment of resident tissue stem cells
- epithelial to mesenchymal transition of the tumor parenchyma
- fibroblast recruitment into the tumor stroma, and
- recruitment of bone marrow-derived cells from the circulation (144-147).

Particular interesting are the evidences pointing to a possible role played by either bone marrow-derived or resident mesenchymal stem cells(148, 149), since mesenchymal stem cells can originate many of the cell types present in the tissue microenvironment, can migrate to site of damages and are able to induce immune-tolerance.

The ***mechanisms that lead to establish a TAF phenotype*** are not clear yet. Fibroblasts are usually activated during injury, but, upon healing, they loose their activated state. In contrast to wound healing, tumor associated fibroblast remain in a chronic state of activation and ultimately support tumor progression. In this regard, Haddow and Dvorak were the first to describe tumors as "wounds that do not heal" (150, 151). The activation can be induced by various factors released during injury

(143), including growth factors such as transforming growth factors TGF-β, epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and fibroblast growth factor 2 (FGF2), which are released from injured epithelial cells and infiltrating mononuclear cells such as monocytes and macrophages (Figure 1.15) (143).

In addition, fibroblasts are activated by direct cell–cell communication and contacts with leukocytes through adhesion molecules such as intercellular-adhesion molecule 1 (ICAM1) or vascular-cell adhesion molecule 1 (VCAM1) (143). Fibroblast activation can also be achieved through reactive oxygen species, complement factor C1 or altered ECM composition (143). Moreover, during tumorigenesis, following loss of basement membrane integrity, tumor cells invade into the underlying connective tissue and interact directly with local mesenchymal fibroblasts (Figure 1.15).

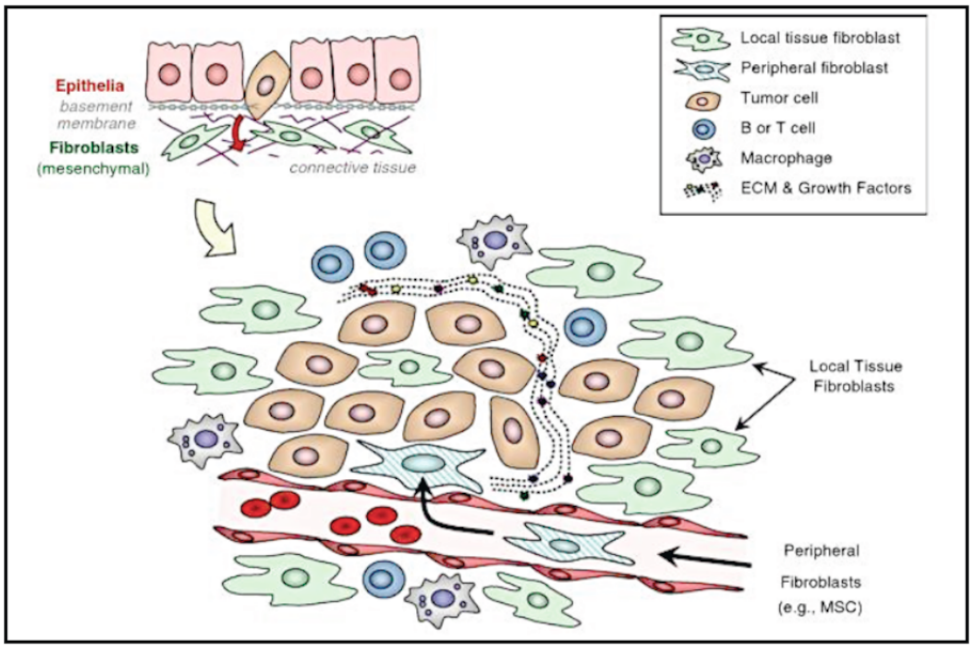


Figure 1. 15. The cross-talk between tumor and stromal cells. Similar to an organ, solid tumor masses are composed of a complex mixture of cellular and acellular components, which together comprise the tumor microenvironment. Following loss of basement membrane integrity, tumor cells invade into the underlying connective tissue and interact directly with local mesenchymal fibroblasts. Subsequent tumor and fibroblast cell expansion promotes recruitment of immune cells, peripheral mesenchymal fibroblasts, and de novo production and remodeling of extracellular matrix (ECM). From (152)

Importantly, the mechanisms that determine a permanent activation of fibroblasts in tumors are still unknown, although epigenetic mechanisms have been hypothesized(128, 138).

The essential role of TAF in the tumor progression suggested the possibility to envision ***TAF as possible novel therapeutic target*** (Table 1.3). The advantages of using the stroma as a therapeutic target comprises, that these cells are not genetically unstable as the cancer cells and they are less likely to develop resistance to treatment and to therapy.

Studeny and collaborators showed that recombinant IFN-β as well as MSC that expressed IFN-β (MSC-IFN-β cells) directly inhibited proliferation of human tumor cells *in vitro.* Importantly, MSC-IFN-β cells that produced IFN-β locally in the tumor microenvironment inhibited malignant cell growth *in vivo,* whereas systemically administered recombinant IFN-β did not. They found that inhibition of tumor cell growth by MSC-IFN-β cells was not permanent(153, 154). This lack of a sustained effect likely reflects the fact that adenoviral trans-genes do not integrate into the genomes of transduced MSC and that transgene copy number per cell declines as MSC-IFN-β cells proliferate in tumors. Sustained inhibition of tumor cell proliferation may be achievable through the use of MSC that are stably transfected with a plasmid that expresses IFN-β under the control of conditional promoter. Even if MSC-IFN-β cells engraft selectively in tumors, the IFN-β they produce could still be released into the circulation and contribute to toxicity by affecting other organs (153, 154).

Table 2 | **Potential therapeutic targets in the tumour stroma**

Stromal element	Alteration	Clinical and preclinical trial results
Stromal cells		
Endothelial cells	Blocking endothelial-cell proliferation (such as through inhibition of VEGF)	Induces endothelial-cell apoptosis, inhibits angiogenesis (TABLE 1) and numerous experimental systems[64]
Tumour-associated fibroblasts	Inhibiting fibroblast proliferation and activation (such as through inhibition of TGFβ signalling)	Promotes tumour progression; TGFβ overexpression, however, can promote malignancy in certain tumour types; tested in preclinical models of colon carcinoma, prostate and forestomach cancer[13,24]
Macrophages	Inhibiting macrophage recruitment by blocking recruitment factors	Reduces tumour malignancy in preclinical models of breast cancer[51,54]
Mast cells	Inhibiting mast-cell recruitment, such as by blocking recruitment factors	Reduces SCC skin tumour malignancy in mast-cell-deficient mice[12,59]
ECM molecules		
Thrombospondin	Overexpression	Inhibits tumour invasion in experimental models for skin SCC and other tumours[76,77]
Tenacin	Inhibition with radiolabelled inhibitory antibodies; inhibition of expression; blockade of tenascin binding inhibits tumour-cell migration	Prolonged patient survival in clinical trials of patients with gliomas (TABLE 2)[114]; inhibits angiogenesis and tumour-cell migration in preclinical models of melanoma and breast cancer[115,116]
Fibronectin	Inhibitory antibodies that target extradomain B	Inhibits angiogenesis in various tumour models[117]
Decorin	Adenovirus-mediated expression	Suppresses tumorigenicity of colon and squamous carcinoma models[118]
Hyaluronate	Expression; degradation by hyaluroniclase	Promotes motility of tumour cells[119]; reduces tumour growth in preclinical models of melanoma and breast cancer[120]
ECM cleavage products	Generation of endostatin, angiostatin, tumstatin and others	Inhibits angiogenesis in various solid tumour models[17,87] and clinical trials (TABLE 2)
Matrix-degrading proteases and inhibitors		
MMPs	Inhibition	Inhibits invasion and angiogenesis, but can also be pro-angiogenic, by inhibiting the generation of reactive ECM fragments; negative results in clinical trials (TABLE 2) involving different treatment combinations[20]
ADAMs	Inhibition	Blocks release of growth factors from the ECM; increased expression observed in human tumour samples and preclinical tumour models[80]
Serpin/PAI1	Altering expression levels	Enzymes are pro-angiogenic at high (therapeutic) concentrations and anti-angiogenic at low (physiological) concentrations in preclinical models of skin SCC and other tumour models[59,80]
TIMPs	Expression	Suppresses tumour invasion and metastasis in tumour models, yet high expression levels correlate with poor prognosis in some human tumour types; expression studies performed in clinical samples and preclinical models[80,88]
Regulatory molecules		
Integrins	Inhibiting signalling	Blocks malignant progression and angiogenesis in clinical trials (TABLE 2) and preclinical models for breast and ovarian cancer[72–74]
Growth factors, cytokines produced by tumour cells (VEGF, PDGF, G-CSF, GM-CSF and others) or stromal cells (TGFβ, CSF1, HGF and IGF1 and others)	Inhibiting signalling	Inhibits tumour progression in preclinical models for colon carcinoma, breast, prostate and forestomach cancer[9,13,24,29,30,54] as well as skin SCC[55,68]
Inflammation-associated growth factors and chemokines	Inhibition	Blocks inflammation, which is associated with poor patient prognosis in clinical and preclinical models[45,51]

ADAM, a disintegrin and metalloproteinase; CSF1, colony stimulating factor 1; ECM, extracellular matrix; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; HGF, hepatocyte growth factor; IGF1, insulin-like growth factor 1; MMP, matrix metalloproteinase; PAI1, plasminogen activator inhibitor 1; PDGF, platelet-derived growth factor; SCC, squamous-cell carcinoma; TGFβ, transforming growth factor-β; VEGF, vascular endothelial growth factor.

Table 1.2 . Potential therapeutic targets in the tumor stroma. From (132).

Another interesting approach is to inhibit TAF by blocking molecules responsible for their activation or those they express. For examples, inhibition of TGF-β by specific molecules seems to reduce the incidence of metastases (155). Another possible target is the Fibroblast Activation

Protein (FAP), thought to be involved in the control of fibroblast growth or epithelial-mesenchymal interactions during development, tissue repair, and epithelial carcinogenesis. Blocking FAP by using sibrotuzumab (a monoclonal antibody directed against FAP), in patients with advanced colorectal carcinoma or non-small-cell lung carcinoma, the effect was tumor-selective without apparent side effects (143). Further study are suggested the use of Bevacizumab, a recombinant, humanized anti–vascular endothelial growth factor monoclonal antibody, able to inhibit tumor angiogenesis (156).
However, the results of these alternative approaches have created, in some cases, disappointment; clinical trials utilizing MMP inhibitors, were not effective in patients with advanced cancer, produced intolerable side effects, and, in some cases, have worsen the prognosis (157). This could be explained by the fact that they MMPs play many roles, from the modulation of cell adhesion to the activation of growth factors. In order to overcome this problem it should be central to achieve greater action selectivity.
In any case, it is very important to notice that this *targeted therapies* are aimed at targeting only the stromal component of the tumor and have not direct effect on the other components of cancer. A rational approach could be combining "stromal therapy" with a cytotoxic approach against tumor cells.

1.4.2 The Glioma Microenvironment

Regarding gliomas, it is becoming apparent the role-played by tumor-associated parenchymal cells, such as vascular cells, microglia, peripheral immune cells, activated astrocytes and neural precursor cells, in defining many of the key-features of brain tumors and in controlling the course of pathology (Figure 1.16) (158).

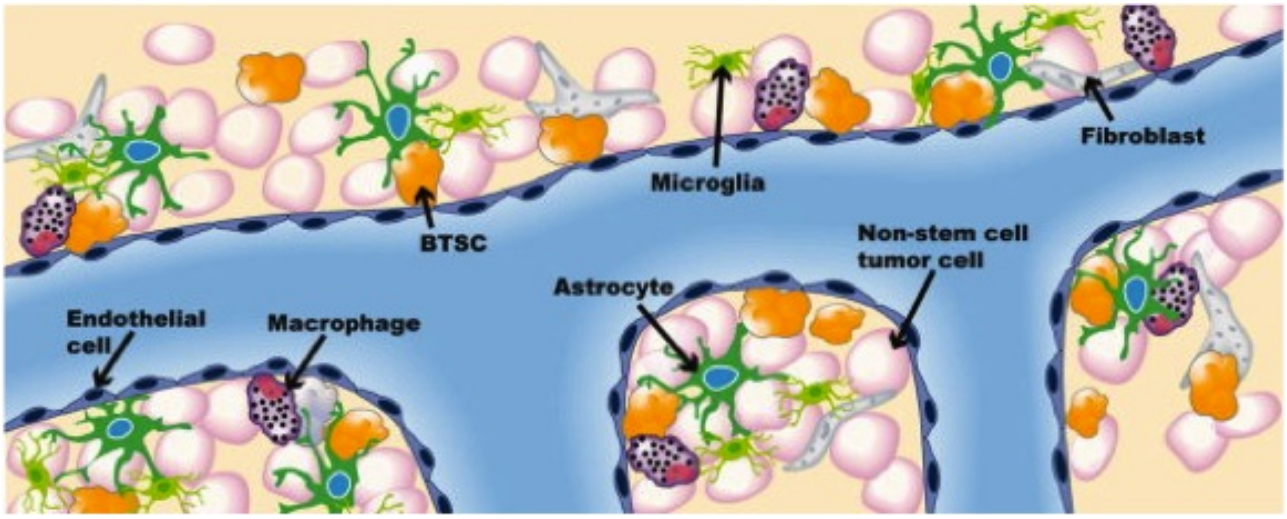


Figure 1. 16. The glioma microenvironment. From (158).

For example, the tumor-vasculature not only supports gliomas, but also provides a specialized niche, named perivascular niche(159-161), fundamental for GSC self-renewal(162). Microglial cells, which can contribute up to 30% of a brain tumor mass, promote glioma migration and tumor growth(163, 164). Non-neoplastic astrocytes can be converted into a reactive phenotype by the glioma microenvironment(165, 166) and can then secrete a number of factors which influences tumor proliferation and invasion, for example via activation of pro-MMP2(167), production of SDF-1(168), neurotrophic factors(169) and astrocyte elevated gene-1 (AEG-1)(170). Importantly, tumor microenvironment not only profoundly controls tumor biology, but also can interfere with therapy. For example, recent data suggests that GSC are neither resistant nor susceptible to chemotherapy per se(171). Together with detoxifying proteins such as O6-methylguanine-DNA-methyltransferase (MGMT), which confers a strong intrinsic resistance to GSC in all studies(49, 50), extrinsic factors may also contribute to the resistance of GSC to temozolomide (TMZ). These may include TMZ concentrations in the brain parenchyma, TMZ dosing schemes, hypoxic microenvironments, niche factors, and the re-acquisition of stem cell properties by non-stem cells(171). It is therefore necessary to consider these factors in order to overcome chemoresistance in the patient(171).

A special issue regards stem cells, other than GSC, present in the brain tumor. For example, the young brain may have the capacity to inhibit gliomagenesis by the endogenous neural stem and progenitor cells that migrate toward primary brain tumors(172-174) and secrete tumor suppressive factors(173-177). In addition, genetic fate mapping studies have shown that reactive glia could acquire a stem cell potential outside the two major neural stem cell niches in adult mammalians (178, 179). Similarly, PDGF-induced gliomas arising in both adult and neonatal rats have been shown to contain normal stem and progenitor cells ‘‘recruited’’ into glioma mass and induced to proliferate, supporting the hypothesis that proliferative stem-like portions of the tumor can arise from normal progenitors(180). However, the precise nature and specific functional characteristics of these ‘‘recruited’’ stem or progenitor cells have not been described in humans(180).

2 AIM OF THE STUDY

Gliomas are the most frequent primitive tumors of the central nervous system. Although relatively uncommon, high-grade gliomas (HGG) are associated with disproportionately high morbidity and mortality. Despite optimal treatment, the median survival is only 12 to 15 months for patients with glioblastoma and 2 to 5 years for patients with anaplastic glioma. With respect to HGG, Low Grade Gliomas (LGG) grow slowly, but about 70% of grade II gliomas evolve to anaplasia within 5-10 years (29). Therefore, despite the increasing knowledge about the molecular biology of CNS cancer, still many important clinical questions are open.

Both in LGG and HGG there is a wide clinical heterogeneity that can be only partly predicted by histological analyses combined with the integration of new discovered markers(26, 28, 37, 47).

Moreover, although chemotherapeutics play an important role in the combined treatment of HGG and progressive LGG, chemotherapy often fails to demonstrate a sustainable beneficial clinical outcome, due to the acquired chemoresistance to drugs such as temozolomide(171).

Therefore novel prognostic and predictive factors are required to improve the correct clinical management of the patients and innovative drugs are mandatory to really impact patient prognosis.

In this regard, in the last years several researchers have suggested the possible usefulness of innovative treatment protocols targeting the glioma-initiating cells, the small population of tumor cells truly responsible for the expansion of the tumor. However, the two methods optimized to isolate glioma stem cells (GSC) presented some important limitations(79, 90, 117-119). The prospective isolation of cells expressing CD133 allows to obtain an extremely variable, but usually low, number of cells, while neurosphere formation is effective only in a fraction of high-grade glioma samples and is time consuming (102). Modifications of the original protocols (181) and optimization of novel procedures (182) apt to overcome these restrictions are now under investigations.

Recently, great interest is focused on the *tumor-associated stroma*. Although constituted by recruited normal cells, it is not a passive spectator but it plays an active role in tumorigenesis. These stroma components have been studied essentially in solid tumor of epithelial origin, where Cancer Associated Fibroblast (CAF) represent the most abundant cell types. Although the biological origin of CAF is still undetermined, evidences point out a possible role played by either bone marrow-derived or resident mesenchymal stem cells (148, 149). Regarding gliomas, xenotransplantation experiments employing human glioma cell lines have suggested that astrocytes in the vicinity of

glioma cells can be activated and facilitate tumor invasiveness (158). In addition, genetic fate mapping studies have shown that reactive glia could acquire a stem cell potential outside the two major neural stem cell niches in adult mammalians (178, 179). Similarly, Platelet Derived Growth Factor (PDGF)-induced gliomas arising in both adult and neonatal rats have been shown to contain normal stem cells and progenitor cells ‘‘recruited’’ into the glioma mass and induced to proliferate, supporting the hypothesis that proliferative stem-like portions of the tumor can arise from normal progenitors (180). However, precise nature and specific functional characteristics of these ‘‘recruited’’ stem or progenitor cells have not been described in humans(180).

Recently, our laboratory has optimized a method to isolate a population of Multipotent Adult Stem Cells (MASC) from several adult human organs (heart, liver and bone marrow) (82, 149, 183). MASC were characterized by a mesenchymal immunophenotype, expressed pluripotent state specific transcription factors, such as Oct-4, Sox-2 and Nanog, and were characterized by a wide differentiation capacity, being able to give rise to derivatives of all the three germ layers. Moreover, in pathological settings we showed that this cells could become an in vitro model of the disease(149).

Therefore, specific aims of the present study were:

1. To isolate, expand and characterize, *in vitro,* a polulation of multipotent adult stem cells, from human low- and high-grade glioma samples
2. To assess whether glioma-derived cell lines display in vitro:
   - stem cell properties
   - tumor-supporting cell properties
3. To evaluate the possibility to correlate some in vitro features/properties of glioma derived cells lines with patient prognosis

3 MATERIALS AND METHODS

3.1 Tissue donors

Human glioma samples were collected by the Neurosurgery Department of the Azienda Ospedaliero Universitaria of Udine, after informed consent was obtained, in accordance with the Declaration of Helsinki, and with approval by the Independent Ethics Committee of the University-Hospital of Udine.

One hundred twenty-two tumor samples were collected from patients with a diagnosis of de novo supratentorial gliomas not previously chemo or radio treated. All patients underwent extensive surgery. Part of the samples were immediately snap frozen and stored at -80°C, part formalin-fixed paraffin embedded, and part freshly used for cell isolation. Tumors were histopathologically classified according to the WHO classification(24). Eligibility criteria included written informed consent, availability of follow-up data. Clinical information was obtained by reviewing the medical records on radiographic images, by telephone or written correspondence, and by review of death certificate. A patient was considered to have recurrent disease if this was revealed either by magnetic resonance imaging or the occurrence of new neurologic symptoms. Patient characteristics are shown in Table 4.1. Patient data were analyzed after a mean follow-up period of 48 months. Extensive surgical resection was done at diagnosis and adjuvant therapy (radiotherapy and/or chemotherapy) was administered in case of tumor recurrence.

3.2 Histological examination.

These experiments were performed in collaboration with S.O.C. of Anatomy Pathology in Udine Hospital. Tumors were histopathologically reviewed by two expert neuropathologists according to WHO (22). Mitotic index was evaluated on Hematoxylin/Eosin stained sections by the number of mitosis in 10 high-power (hpf). Ki-67 (1:200), clone, Mib-1, Dako), GFAP (1:400, clone 6F2, Dako), EGFR (1:50, clone 31G7, Zymed), p53 (1:200, clone D07, Dako) and IDH1 ^{R132H} (1:150, clone H09, Dianova) were detected by immunohistochemistry on 4µm-thick FFPE (Formalin-Fixed Paraffin-Embedded) sections. Ki-67 was scored as percentage of positive nuclei. All other markers were qualitatively evaluated as negative or positive. FISH analysis for 1p36 and 19q13 deletions was performed on 4µm-think FFPE sections using dual-color 1p36/1q25 and 19q13/19p13 probes (Vysis, Abbott). IDH gene status and MGMT (06-methylguanine-DNAmethyltransferase) promoter methylation were assessed on DNA extracted from FFPE tissue (QIAmp DNA mini kit-Qiagen).

IDH1 and IDH2 gene status was evaluated by pyrosequencing as in (184).After DNA bisulfite conversion (EpiTect Bisulfite Kit-Qiagen), methylation levels of the MGMT promoter in position 17-39 of exon 1 were investigated by PyroMark Q96-CpG-MGMT (Qiagen) according to manufacturers' instructions.

3.3 Volumetric Analysis

All pre- and post-operative tumoral segmentations were performed manually across all MRI (Magnetic Resonance Imaging) slices with the OSIRIX software tool to measure tumor volumes (cm^3) on the basis of T2 axial slices, as in (185, 186). The extent of resection (EOR) was evaluated by using Magnetic Resonance Imaging (MRI) images acquired four months after surgery. EOR was calculated (185, 186) : (pre-operative tumor volume - post-operative tumor volume)/ pre-operative tumor volume.

3.4 Glioma Associated Stem cell (GASC) isolation and culture

Cells from glioma were isolated and cultured applying, with minor modification, a protocol optimized for culturing multipotent adult stem cells from normal(21, 23) and neoplastic tissues(16). Briefly, glioma sample fragments were first disaggregated mechanically with scalpels and then enzimatically dissociated in a 0.0125% Collagenase type II solution (Worthington) in Joklik modified Eagle's Medium (Sigma-Aldrich) for 5 minutes at 37°C. Collagenase activity was stopped by the addition of 0.1% BSA (Sigma-Aldrich) solution in Joklik modified Eagle's Medium (Sigma-Aldrich). Cell suspension was centrifuged at 500g for 10 minutes and filtered through a sieve (BD Falcon) in order to select a population less than 40 μm in diameter.
$2.0x10^6$ freshly isolated human cells were plated onto 100 mm human fibronectin (Sigma-Aldrich) coated dishes (BD Falcon) in an expansion medium composed as follows: 60% low glucose DMEM (Invitrogen), 40% MCDB-201, 1mg/mL linoleic acid-BSA, 10-9 M dexamethasone, 10-4 M ascorbic acid-2 phosphate, 1X insulin-transferrin-sodium selenite (all from Sigma-Aldrich), 2% fetal bovine serum (StemCell Technologies), 10 ng/ml human or murine PDGF-BB, 10 ng/ml human or murine EGF (both from Peprotech EC). Medium was replaced with fresh one every 4 days. Once cells reached 70-80% of confluence, they were detached with TrypLE Express (Invitrogen) and re-plated at a density of 1-2 $x10^3/cm^2$.

3.5 Cell growth kinetic

Cells were seeded at a density of 2000 cells/cm2 in expansion medium. Medium was replaced with fresh one every 4 days and, at different time points (1-2-3-5-7 and 10 days), cells were detached with TrypLE Express (Invitrogen) and counted.

3.6 Single cell cloning

To generate single- cell–derived clones, a cell sorter (MoFlo, Beckman-Coulter) was used to automatically deposit individual GASC at the third passage in culture into fibronectin-coated wells of 96well Terasaki plates (n=1337 wells) and cultured in expansion medium added with 10%FBS. To determine sorting efficiency and to verify if any well was seeded with more than one cell, we utilized the Vybrant CFDA SE (CFSE) as a cell tracker (Molecular Probes, Invitrogen). Wells were examined once a week. When clones reached confluence, cells were detached utilizing TrypLE (Invitrogen) and plated at the density of 1500 cells/cm2 on new fibronectin- coated dishes and cultured in expansion medium.

3.7 Induction of in vitro neural differentiation

GASC were differentiated and analyzed as in (21). Briefly, 3.000 cells/cm2 were plated in DMEM-high glucose (Invitrogen, Carlsbad, CA, USA), 10% FBS (Sigma-Aldrich, st. louis, MO, USA). After 24 h medium was replaced with DMEM-high glucose, 10% FBS containing B27 (Invitrogen, Carlsbad, CA, USA), 10 ng/ml EGF and 20 ng/ml bFGF (both from Peprotech EC, London, UK). After 5 days, cells were washed and incubated with DMEM containing 5 μg/ml insulin, 200 μM indomethacin and 0.5 mM IBMX (all from Sigma-Aldrich, St. loius, MO, USA), in the absence of FBS for 5-10 days. At the end of every treatment, cells were fixed with 4% buffered Paraformaldehyde, and assessed for the expression of neuronal, glial and oligodendroglyal markers.

3.8 Soft agar assay

To evaluate the ability of freshly isolated glioma cells and of GASC, to grow in an anchorage-independent way, 50.000 cells were plated in a 0.25% soft agar solution in 35-mm plates containing a basal layer of 1% agarose; colonies were counted after 2-3 weeks under a phase contrast microscope (Leica DMI6000B). Assays were performed in triplicate. For the comparison of U87-CM and A172-CM, colonies formation were analyzed 2 weeks after seeding by acquiring at 10X, using the Leica DMI-6000B, a series of optical sections obtained by scanning along the z-axis field

of interest. Once obtained the sum image, it was assessed the number of colonies with a diameter greater than 60 microns. For each replicate, a volume of 500µm3 has been sampled.

3.9 Conditioning of A172 and U87 glioma cell lines with semi-conditioned medium (CM) from GASC.

One day after seeding into T75 flasks (2 × 106 cells), low-grade glioma GASC (n=3) were washed twice with serum-free HBSS (Sigma-Aldrich), and then incubated for 24 h with serum-free DMEM supplemented with 2 mM L-glutamine (both from Sigma-Aldrich) (15 mL/T75). Medium was then collected, filtered through a 0.3µm filter (Millipore) and kept at +4°C. Serum-free DMEM supplemented with 2 mM L-glutamine incubated for 24 h in cell culture flasks without cells was used as control.

For conditioning experiments, conditioned media obtained from low-grade glioma GASC and empty flask, were diluted 1:1 with DMEM supplemented with 2 mM L-glutamine and 20% fetal bovine serum (Gibco) in order to obtain GASC-CM and non-CM, respectively.

A172 (25) and U87 MG (26) were cultured in GASC-CM and non- CM for 2 passages (≈8 population doublings) and finally analyzed in terms of population doubling time, surface immunophenotype, and assayed in migration and soft-agar assays. All experiments were performed in triplicate.

3.10 Reverse transcriptase PCR analysis

Total RNA was extracted from non-confluent cultures of GASC at P3 using the TRIzol Reagent (Invitrogen). After treatment with DNase I (Ambion), first strand cDNA synthesis was performed with 1 µg total RNA using random hexanucleotides and MMLV reverse transcriptase (Invitrogen). PCR amplification was carried out in a final volume of 50 µl, using 80-150 ng cDNA, 10 mM Tris-HCl pH 9.0, 1.5 mM MgCl2, 0.2 mM dNTPs, 25 pmol of each primer and 2U Taq I polymerase (Amersham). The PCR conditions were as follows: 94°C for 2 minutes; 40 cycles at 95°C for 30 sec, 61°C for 60 sec and 72°C for 60 sec. The optimal conditions and the number of cycles were determined to allow amplification of samples within the linear phase of the PCR. The reaction products were analyzed on 3% agarose gels. Primer pairs and product length were the following: OCT4 reference sequence ENST00000259915 primer FW 5’CGAAAGAGAAAGCGAACCAGTAT3’ and primer RW 5’CGAGAGGATTTTGAGGCTGCT3’ (product lenght 216bp); SOX2 (SRY-box2 reference sequence ENST00000325404 primer FW 5’ATGGGTTCGGTGGTCAAGT 3’ and primer RW 5’CCTGTGGTTACCTCTTCCTCC 3’ (product length 60bp); NANOG reference sequence ENST00000229307 primer FW

5’ATGCCTCACACGGAGACTGT 3’ and primer RW 5’TGCTTATTCAGGACAGCCCT3’ (product length 66bp); GAPDH (glyceraldehyde-3-phosphate dehydrogenase) reference sequence ENST00000229239 primer FW 5’ACCCACTCCTCCACCTTTGACG 3’ and primer RW 5’AGCAAGAGCACAAGAGGAAGAGAGA’3 (product length 187bp); KLF4 (KRUPPEL-LIKE FACTOR-4 (GUT)) reference sequence NM_004235.4 primer FW 5’GGGAGAAGACACTGCGTCA 3’and primer RW 5’GGAAGCACTGGGGGAAGT 3’(product lengh 88pb); MYC reference sequence NM_002467.4 primer FW 5’GCTGCTTAGACGCTGGATTT 3’ and primer RW 5’TAACGTTGAGGGGCATCG 3’ (product lengh 73pb

3.11 Scratch assay

In order to evaluate in vitro cell migration of A172 and U87-MG conditioned, or not, by glioma GASC, a scratch assay was performed (27). In 33mm-plate at high confluence, scratches were created utilizing 200µl tips. Phase contrast images of the scratches were acquired at 3-hour intervals, until their complete closing, utilizing Leica DMI6000B. Images were then compared and quantified by ImageJ in order to calculate the rate of cell migration.

3.12 Flow-cytometry

Proliferating cells were detached with 0.25% trypsin-EDTA (Sigma-Aldrich) and, after a 20 minutes recovery phase, were incubated with either properly conjugated primary antibodies: CD13, CD29, CD49a, CD49b, CD49d, CD90, CD73, CD44, CD59, CD45, HLA-DR, CD117, CD271, CD34, (BD Biosciences), CD105, CD66e (Serotech), CD133 (Miltenyi Biotec), E-cadherin (Santa Cruz Biotechnology), ABCG-2 (Chemicon International), or with an unconjugated primary antibody: N-cadherin (Sigma-Aldrich). Unconjugated antibody was revealed using PE or FITC conjugated secondary antibodies (DakoCytomation). Properly conjugated isotype matched antibodies were used as a negative control. The analysis was performed either by FACS-Calibur (BD Biosciences) or by CyAn (Dako Cytomation).

3.13 Immunofluorescence and histochemistry.

Cells cultured either in expansion or in differentiation medium were fixed in 4% buffered paraformaldehyde for 20 minutes at room temperature (R.T.). For intracellular stainings, fixed cells were permeabilized for 10 minutes at R.T. with 0.1% Triton X-100 (Sigma-Aldrich) before exposing them to primary antibodies. In order to block unspecific binding of the primary antibodies, cells were incubated with 10% donkey serum in PBS for 30 min. Primary antibody incubation was

performed over-night at 4°C using following dilutions: Oct-4 (Abcam, 1:150); Sox-2 (Chemicon, 1:150); Nanog (Abcam, 1:150); ß3-tubulin (Abcam, 1:1000); Smooth Muscle Actin (Dako, 1:50); GFAP (Dako, 1:50); O4 (Millipore, 1:100); synaptophisin (Dako, 1:100). For the following antibodies the incubation was performed one hour at room temperature: Vimentin (Dako 1:500), and VEGF (Neomarkers 1:100). To detect primary antibodies, A488 and A555 dyes labeled secondary antibodies, diluted 1:800, were employed (Molecular Probe, Invitrogen). Cells were incubated with the secondary antibody diluted in PBS for 1 hour in a humidified chamber at 37°C. Finally, 0.1 µg/ml DAPI (Sigma) was used to identify nuclei. Vectashield (Vector) was used as mounting medium. Confocal image acquisition was carried out by a Confocal Laser Microscope (Leica TCS-SP2, Leica Microsystems) utilizing either a 63x oil immersion objective (numerical aperture: 1.40) or a 40x oil immersion objective (numerical aperture: 1.25). Epifluorescence and phase contrast images were obtained utilizing a live cell imaging dedicated system consisting of a Leica DMI 6000B microscope connected to a Leica DFC350FX camera (Leica Microsystems, Wetzlar, Germany). 10X (numerical aperture: 0.25), 40X oil immersion (numerical aperture: 1.25), and 63X oil immersion (numerical aperture: 1.40) objectives were employed for this purpose. Bright field images were captured utilizing a Leica DMD108 microscope (Leica Microsystems). 10X (numerical aperture: 0.40), 20X (numerical aperture: 0.70), and 40x (numerical aperture: 0.95) objectives were employed. Adobe Photoshop software was utilized to compose, overlay the images and to adjust contrast (Adobe, USA).

3.14 Statistical analysis

Characteristics of the study population are described using standard methods. In order to define a risk profile for LGG patients we adopted a multi step approach (Figure 4.9).

Using De Long's nonparametric ROC analysis we first identified the GASC-features distinguishing LGG from HGG, and, for the most significant ($p<0.005$) parameters, we found the cut-off values able to discriminate, with high sensitivity and specificity, the two populations. Then we expressed them as a binary value (0 or 1 considering whether the variable fell in the range characterizing the LGG or HGG population), and we inserted them in an additive score.

To test the capacity of the score to prognostically stratify LGG patients we analyzed OS (overall survival), PFS(progression-free survival) and MPFS (malignant-progression free survival), defined as follow: OS=time between initial surgery and death; PFS=time between initial surgery and demonstration of unequivocal increase in tumor size on follow-up imaging, malignant progression,

and/or death; MPFS=time between initial surgery and demonstration of gadolinium enhancement on follow-up imaging and/or higher-grade tumor on subsequent biopsy or death.

OS, PFS and MPFS were described using the Kaplan-Meier approach and the log-rank statistic with Bonferroni correction was computed. Analysis of survival was done using Cox proportional hazard models (Table 4.6). Covariates with $p<0.05$ at univariable analysis were then selected for multivariable stepwise analysis. Results are presented as hazard ratios (HR) and 95% confidence intervals (95% CI).

4 RESULTS

4.1 Isolation of proliferating cell lines from low - and high-grade gliomas

In order to establish whether human gliomas possess a population of Glioma Associated Stem cells (GASC), we applied the method, previously optimized to isolate multipotent adult stem cells from normal (82, 149, 183) and neoplastic tissues (149), to *n*=122 de novo supratentorial glioma samples. They have been included in the study patients with a histological diagnosis of low-grade (n=49) or high-grade astrocytoma (n=73), placed by expert pathologists.

GRADE	*N*	FEMALE N (%)	AGE_YEARS (MEAN±ST. DEVIATION)	HISTOPATOLOGICAL DIAGNOSIS
LOW GRADE	49	17 (33%)	34⁀16	Diffuse astrocytoma (n=29) Oligoastrocytoma (n=16) Gemistocytic astrocytoma (n=3) Xantoastrocytoma pleomorfo (n=1)
HIGH GRADE	73	32 (44%)	59⁀12	Glioblastoma multiforme (n=56) Anaplastic astrocytoma (n=9) Gliosarcoma (n=5) Giant cell glioblastoma (n=3)
p	-		*p<0.0001*	

Table 4. 1 Clinical characteristics of the patients included in the study.

Specifically, patients, not previously treated with neo-adjuvant therapy, underwent surgical resection of the tumor at the Neurosurgical Department of the University Hospital of Udine, in the period between June 2006 and March 2011.

In line with epidemiological data, age of patients was significantly different in the group of patients with a high-grade lesion compared to those with a low-grade lesion, being these latter subjects younger (59±12 vs 34±16; $p<0.0001$). With regard to the sex, there was a prevalence of presentation in male subjects in both low- and high-grade glioma patients.

As previously described for normal and neoplastic tissues (82, 149, 187), tissue fragments were digested by collagenase and cells less than 40µm in diameter were cultured on fibronectin-coated dishes in a medium selective for the growth of human multipotent adult stem cells. Flow cytometric analysis documented that freshly isolated, unselected glioma cell population was heterogeneous and included a small fraction of hematopoietic CD45+ cells, while the majority of the CD45- cells expressed the mesenchymal epitopes CD59, CD90, CD73, CD44 and CD271 (Figure 4.1 and Table 4.2).

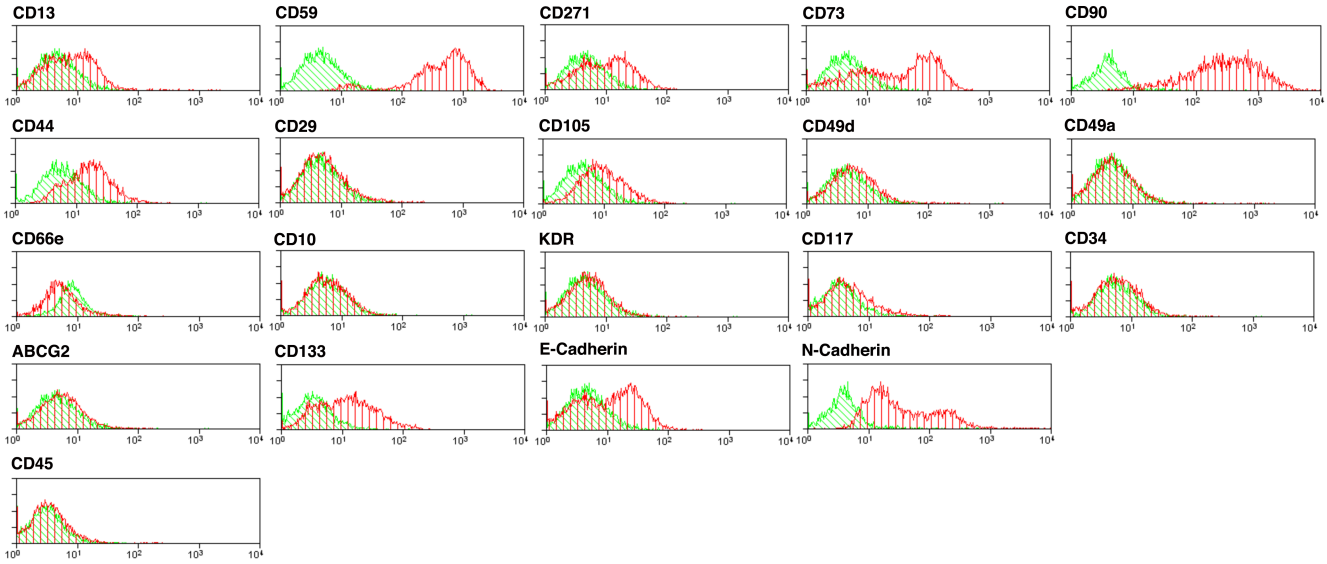


Figure 4.1 . FACS analysis of freshly isolated glioma cells. Green histograms represent isotype-matched stained cells, while red histograms represent cells stained with antibodies directed versus the specified antigen.

Interestingly, low- and high-grade glioma sample significantly differed in the expression of KDR, CD66e and E-Cadherin, being these antigens more expressed in low-grade glioma samples (Table 4.2). Conversely, high-grade glioma derived samples were significantly enriched in endothelial CD34+ cells (Table 4.2). Regarding the presence of cells expressing the glioma stem cell marker CD133, they were represent, as previously described, both in low- and high- grade glioma samples, an extremely variable fraction.

	Low-grade N=7			High-grade N=7			L vs. H (*p* value)
	median	25°p	75°p	median	25°p	75°p	
CD45	15.1	0.8	25.4	4.2	0.6	04.02.00	n,s
Immunophenotype of the CD45$^-$ cell population							
CD59	97.0	96.9	97.9	84.7	34.54	88.2	0,01
CD90	85.4	48.4	88.5	88.4	23.6	95.3	n.s
CD73	78.8	60.5	83.5	20.5	2.4	43.7	0,01
CD44	64.9	22.1	91.1	43.4	22.8	56.3	n.s
CD271	65.2	5.0	70.9	4.1	1.1	58.1	n.s
KDR	56.5	30.4	66.0	11.0	1.7	15.3	0,1
N-CAD	51.8	47.6	68.6	29.9	3.9	63.3	n.s
CD66E	50.3	4.4	61.5	8.7	4.7	23.3	n.s
E-CAD	47.1	7.2	61.3	8.5	1.2	16	n.s
CD49D	26.2	10.9	52.7	8.3	2.1	16.9	n.s
CD29	23.5	14.5	61.6	10.8	1	53.5	n.s
HLA-DR	20.2	0.1	70.4	11.0	4.5	16.9	n.s
CXCR4	13.4	0.03	13.9	4.4	3.0	10.0	n.s
CD13	12.4	2.1	52.8	6.5	1.1	21.4	n.s
CD38	0.09	0.01	0.2	0.03	0	1.7	n.s
CD49A	2.5	1.4	14.7	2.6	1	13.3	n.s
CD117	1.5	1	3.8	0.3	0.05	1.5	n.s
CD105	4.1	0.3	6.1	2.5	1.8	3.1	n.s
CD133	3.2	0.2	17.9	2.5	0.5	40.5	n.s
CD10	1.3	0.3	2.9	0.5	0.1	0.9	n.s
ABCG2	4.0	1.1	7.0	2	0.6	6.6	n.s
CD34	0.1	0.05	0.3	0.5	0.2	1	0,02

Table 4. 2. FACS analyses of freshly isolated cells.

Once cultured in the selective proliferation medium, only a minority of the seeded cells was able to adhere and proliferate (Figure 4.2), and the colony forming efficiency was significantly lower for GASC obtained from high grade gliomas (H) with respect to those obtained from low grade gliomas (L) (54±13 vs 23±5 colonies/10^5 seeded cells, $p<0.05$).

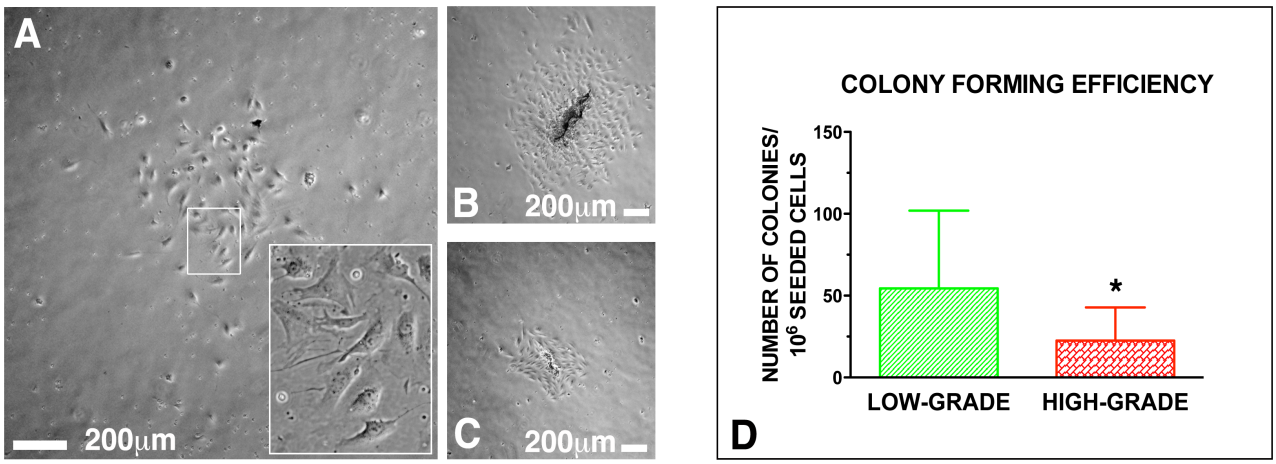


Figure 4. 2. (A-C) Phase contrast images of colonies formed during primary culture of glioma cells in culture conditions selective for the growth of multipotent adult stem cells. (D) Quantification of the colony forming efficiency. Results are espressed as mean ± standard deviation, *, p<0.05 vs. low-grade.

Despite the extremely selective culture conditions, 5-7 days after the primary culture, proliferating cell lines were obtained with an effectiveness of about 95% both from low-grade and high-grade gliomas confirming the high efficiency of the optimized method in obtaining cell lines from both normal and tumor samples (Figure 4.3) (82, 149, 187).

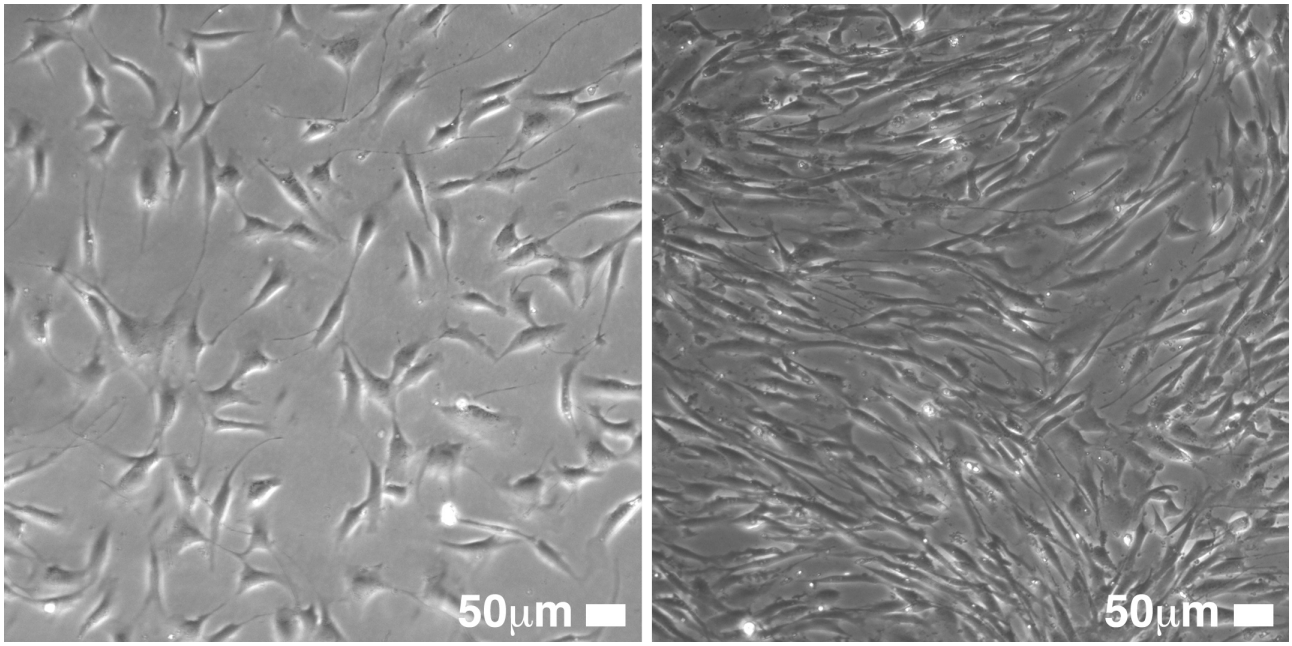


Figure 4. 3. Phase contrast images of primary culture obtained from low grade LGG (A) and HGG (B)

Similarly to what previously shown for normal tissues, proliferating cells were, since the beginning, highly positive for the expression of pluripotent state specific transcription factors and other stem cell markers, such as nestin, excluding that the acquisition of these features could be related to an extensive culture manipulation (Figure 4.4).

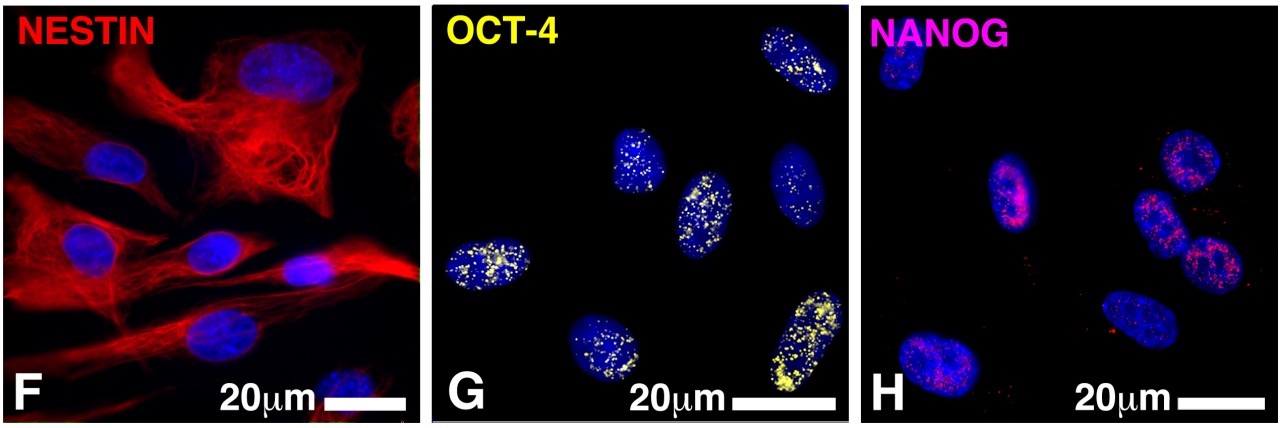


Figure 4.4 . Stem cell marker expression in primary culture. Expression of vimentin (red fluorescence), Oct-4 (yellow fluorescence) and Nanog (magenta fluorescence) 5-7 days after primary culture. Nuclei are depicted by the blue fluorescence of DAPI staining.

4.2 Cell lines associated with low- and high-grade gliomas (GASC) displayed stem cell properties.

After three to four passages in expansion medium, L- and H-GASC displayed a homogeneous, fibroblast-like morphology (Figure 4.5 A-B) and continued to express, both at protein and mRNA level, Oct-4, Nanog and Sox-2, transcription factors considered to be crucial, together with Klf-4 and c-Myc, for the maintenance of embryonic stem cell self-renewal and pluripotency(188) (Figure 4.5 C-J).

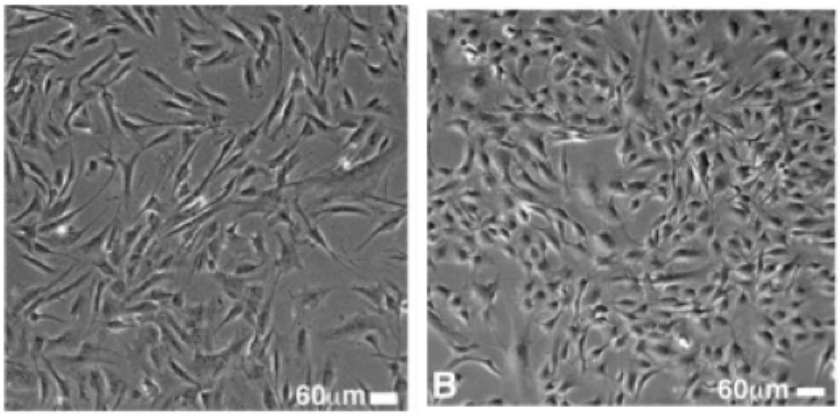


Figure 4.5. (A-B). Phase contrast image of GASC obtained from human, low-(A) and high grade gliomas (B) at the third passage in culture.

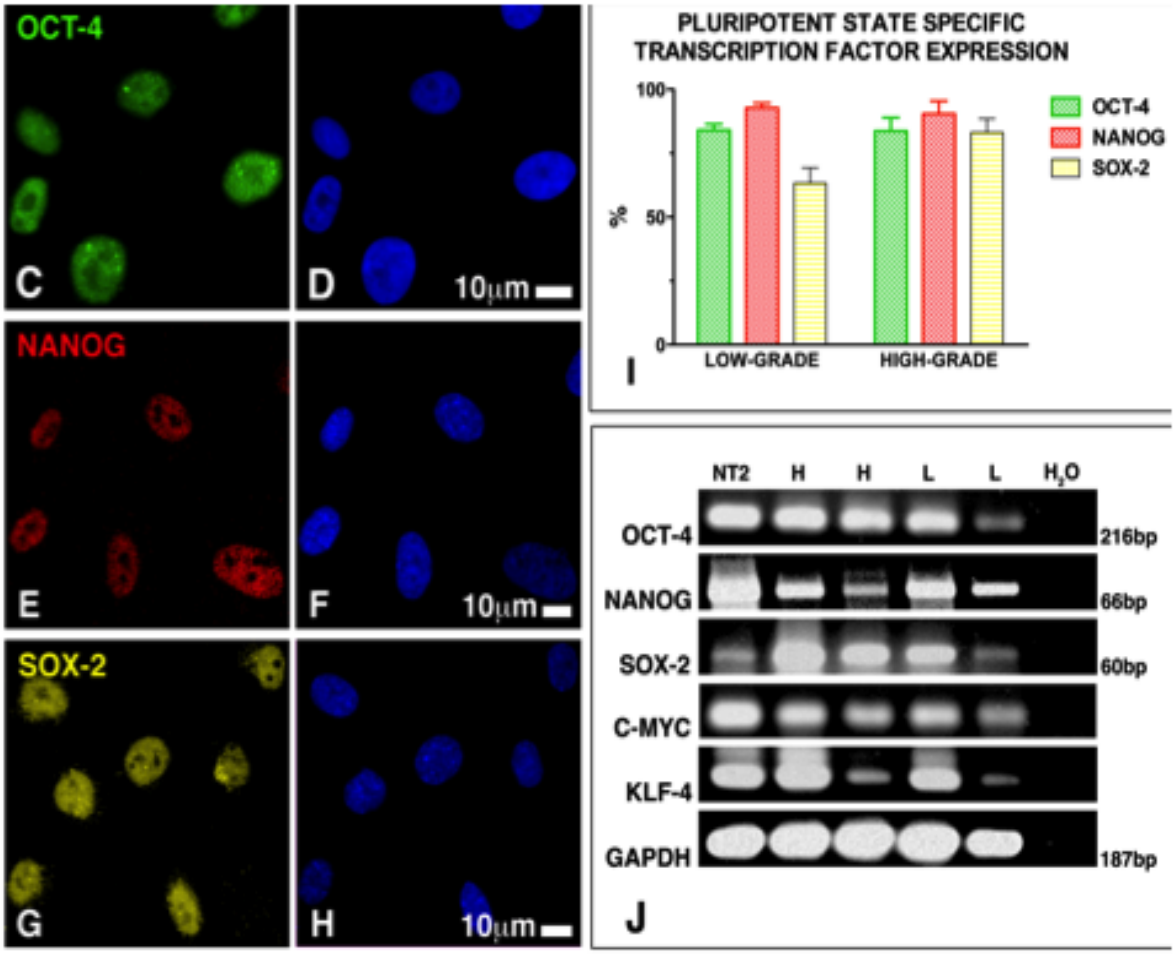


Figure 4. 5 (C-J). Pluripotent state-specific transcription factor (Oct-4, Nanod,Sox-2) expression in GASC. Green fluorescence represent the nuclear expression of Oct-4, in (C-D), red fluorescence represent the nuclear expression of Nanog and in (E-F), yellow fluorescence represent the nuclear expression of Sox-2 proteins (G-H), respectively. In D, F and H, nuclei are depicted by the blue fluorescence of DAPI staining. (I) Results are presented as mean ⁓ standard deviation (J) Representative RT-PCR analysis of Oct-4, Nanog and Sox-2, Kif-4, c-Myc and GAPDH in GASC.

Moreover, cells were highly positive for early intermediate filaments, such as vimentin and nestin, while GFAP was expressed only in a minority of the cells. With respect to L-GASC, H-GASC were significantly more positive for the expression of neuron-specific enolase (Figure 4.5 K-P).

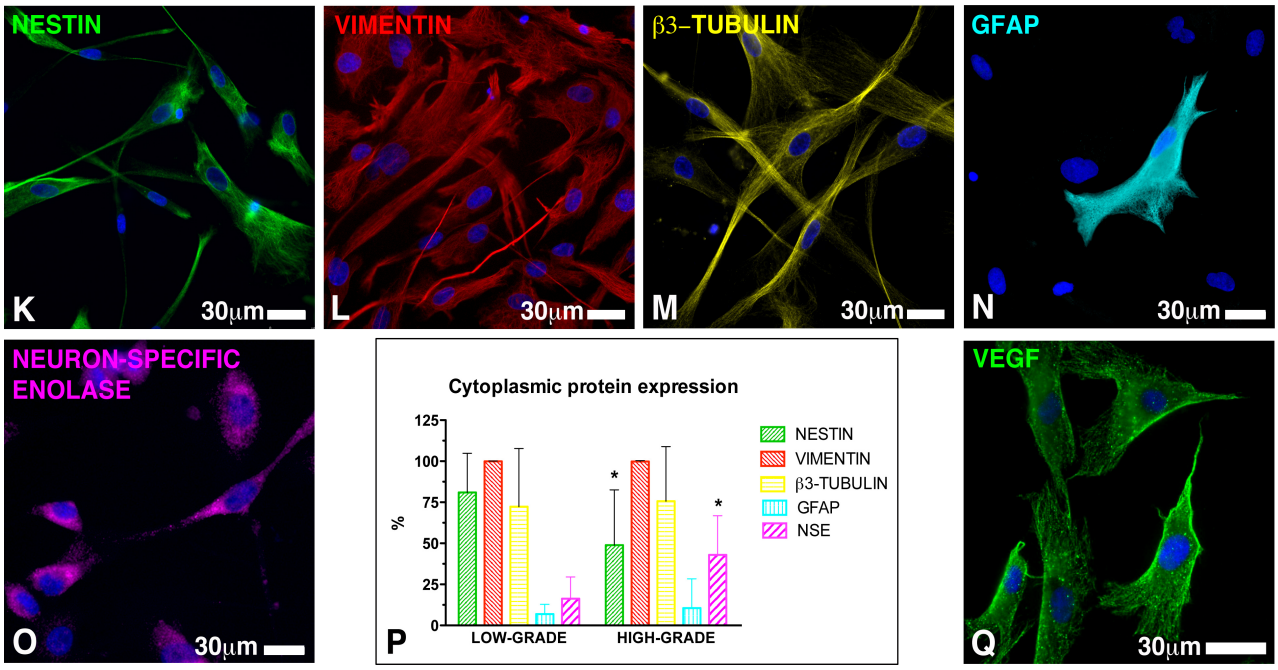


Figure 4. 5 (K-Q). Cytoplasmic protein expression in GASC. Nestin (green fluorescence, K), vimentin (red fluorescence, L), beta 3 tubulin (yellow fluorescence, M), GFAP (cyan fluorescence, N), neuron specific enolase (magenta fluorescence, O) in GASC. nuclei are depicted by the blue fluorescence of DAPI staining. (P) Results are presented as mean ⁓ standard deviation. *, p<0.05 vs LGG. (Q) VEGF expression by GASC (green fluorescence).

Considering the surface immunophenotype, as evaluated by flow-cytometry, L- and H-GASC shared a similar mesenchymal phenotype (Figure 4.5 R) although some significant differences could be detected (Table 4.3). Specifically, H-GASC, with respect to L-GASC, were characterized by an increased expression of CD90, ABCG-2, CD66e and by a significant reduction in CD105 (Table 4.3).

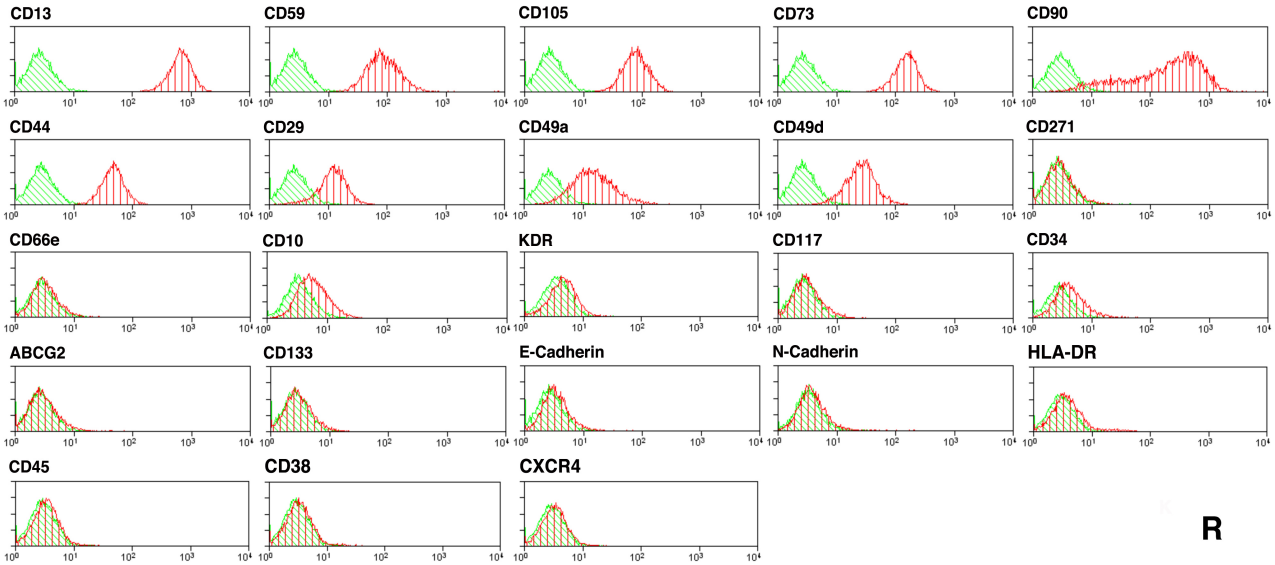


Figure 4. 5. (R) Representative surface immunophenotype of GASC. Histograms overlays show isotype control IgG staining profile (red histograms) versus specific antibody staining profile (green histograms).

	mean±SD						L vs. H
	L-GASC N=43			H-GASC N=19			(*p* value)
	99.01.0						
CD59	0	±	2.0	98.8	±	2.5	n.s
CD73	96.4	±	11.7	96.7	±	3.7	n.s
CD13	91.9	±	24.8	99.3	±	0.9	n.s
CD44	93.0	±	16.4	76.6	±	29.7	0,0004
CD105	86.2	±	29.2	48.7	±	43.3	0
CD49A	84.0	±	25.8	76.6	±	20.2	n.s
CD49D	79.9	±	26.7	71.8	±	28.2	n.s
CD29	69.5	±	28.06.00	67.6	±	28.1	n.s
CD90	69.1	±	26.6	87.2	±	21.1	0,02
CD10	41.1	±	40.1	33.7	±	28.2	n.s
HLA-DR	0.9	±	2.2	0.2	±	0.3	n.s
CD117	14.5	±	24.4	2.5	±	2.5	n.s
N-CAD	11.0	±	22.9	10.1	±	15.2	n.s
E-CAD	5.6	±	12.1	20.7	±	27	0,005
KDR	2.2	±	2.3	21.1	±	23.4	0,04
CD271	1.2	±	4.8	1.1	±	1.3	n.s
ABCG2	1.7	±	1.3	3.5	±	3.1	0,002
CD34	0.9	±	1.1	2.8	±	6.2	n.s
CD66E	1.4	±	3.3	15.1	±	23.9	0,0014
CD45	0.7	±	1.2	0.8	±	0.9	n.s
CXCR4	0.6	±	1.0	1.0	±	0.7	n.s
CD38	0.6	±	0.9	0.4	±	0.6	n.s
CD133	0.5	±	0.9	1.5	±	1.8	0,005

Table 4. 3. Surface immunophenotype of LGG- and HGG-derived GASC.

Considering the growth kinetic, H-GASC did not differ significantly from L-GASC, being the population doubling time 34±18 hours vs 37±13 hours, respectively ($p>0.05$). Importantly, L- and H-GASC behaved as finite cell lines able to proliferate for more than 40 population doublings before reaching cell senescence and growth arrest (data not shown). Only one high-grade glioma cell line underwent a spontaneous immortalization and could be amplified for more 200 doublings without reaching senescence (data not shown).

In order to test whether GASC were characterized by stem cell properties, a single cell cloning assay was performed (n=2 L-GASC and n=2 H-GASC). FACS-sorted individual GASC were deposited in single wells of Terasaki plates. Wells with more than one cell were excluded. Of 1337 seeded wells, GASC gave rise to n=278 highly proliferating clones that could continue to replicate undergoing an additional 20 population doublings without reaching replicative senescence (Figure 4.6). Thus, the actual cloning efficiency of L- and H-GASC was 30⁀4% and 20⁀12%, respectively.

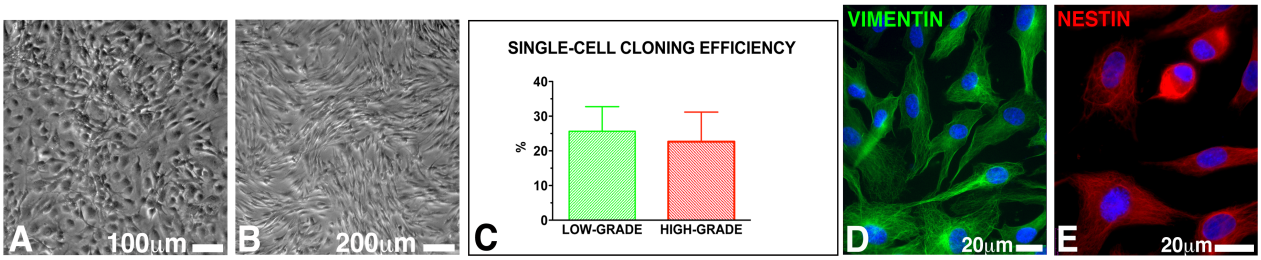


Figure 4. 6. GASC clonogenicity. (A-B) Phase contrast images of single-cell derived clones of LGG- and HGG-derived cells. (C) Results are presented as mean ± standard deviation. (D-E) Vimentin (green fluorescence, D) and nestin (red fluorescence, E) expression by GASC. Nuclei are depicted by the blue fluorescence of DAPI staining.

Importantly, clonal cells maintained a stable phenotype and continued to express nestin (Figure 4.6 D), vimentin (Figure 4.6 E), neuron-specific enolase and the pluripotent state specific transcription factors OCT-4 and NANOG.

In order to verify whether clones derived from GASC were multipotent, cell lines were cultured under appropriate differentiation inducing conditions (see Materials and Methods), and the acquisition of neuronal, glial and oligodendroglial markers was assessed (Figure 4.6 F-P). Cells exposed to neural differentiation medium displayed a morphological change (Figure 4.6 F) and lost the expression of pluripotent state specific transcription factors (Figure 4.6 G, H and O). Specifically, 27±5% of the cells became positive for the neuronal specific marker MAP-2 (Figure 4.6 K, P), while 22±6% of the cells acquired the expression of the glial specific marker GFAP (Figure 4.6 M, P). Importantly, 18±3% of the cells acquired also the ability to differentiate into O4-positive oligodendrocyte-like cells (Figure 4.6 N, P).

Altogether, the accumulated evidences showed that, similarly to what has already been demonstrated for non neoplastic adult human tissues, it is possible to isolate from glioma samples a population of cells characterized by a mesenchymal stem cell phenotype, clonogenicity and by in vitro multipotency.

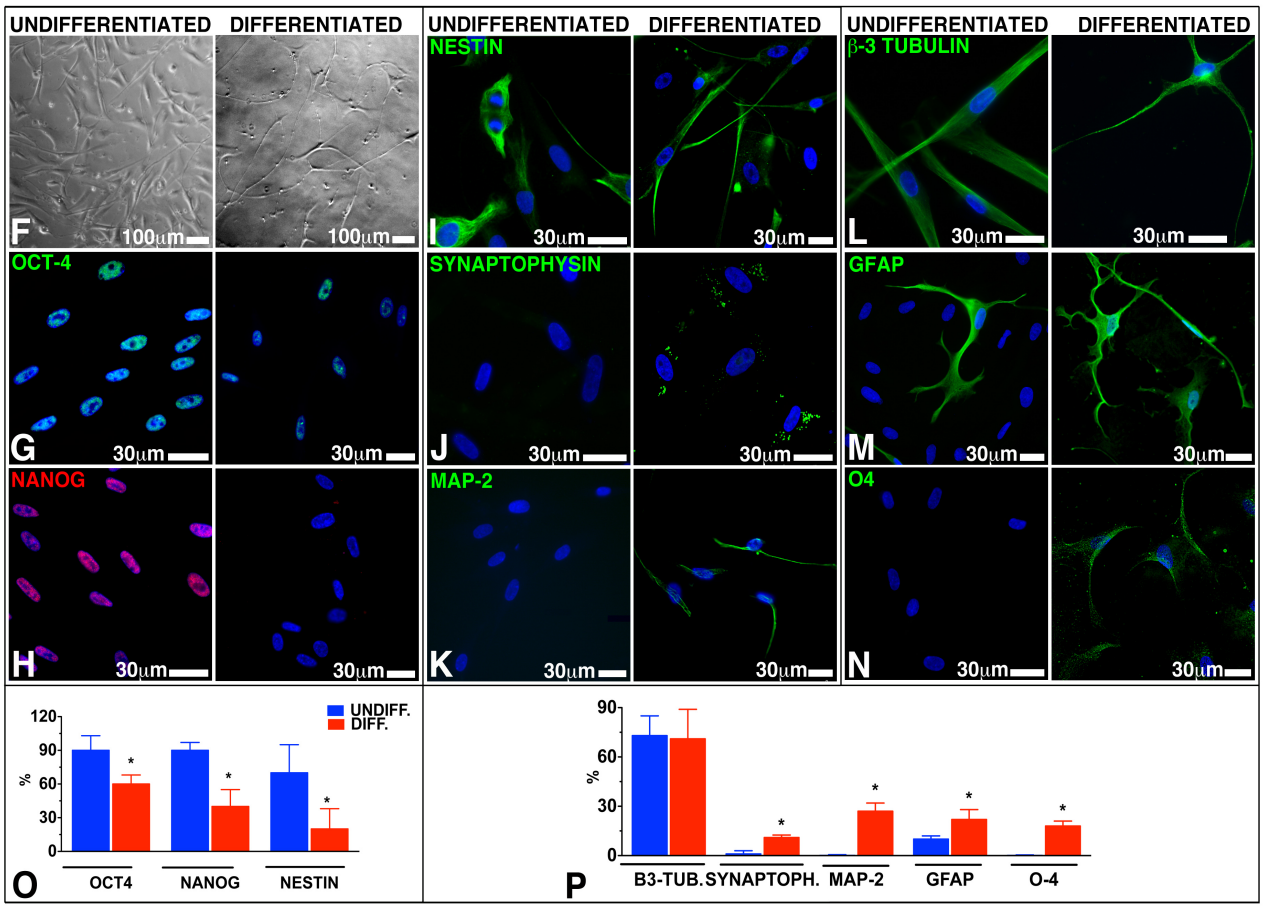


Figure 4. 6 (F-P) Multipotency of GASC-derived single cell derived clones. (F) Phase contrast pictures of undifferentiated GASC (left) and GASC differentiated into neural derivatives (right.). (G-N) Oct.4 (green fluorescence, G) and Nanog (red fluorescence, H), nestin (green fluorescence, I), synaptophisin (green fluorescence, J), MAP-2 (green fluorescence, K), beta3-tubulin (green fluorescence, L), GFAP (green fluorescence, M) and O4 (green fluorescence, N) expression in undifferentiated- (left panels) and differentiated- (right panels) GASC. Nuclei are depicted by the blue fluorescence of DAPI staining. (O-P) Results are expressed as mean ~ standard deviation. *, p<0.05 vs. undifferentiated.

4.3 Glioma-associated stem cells possess aberrant growth properties but are not tumorigenic.

Despite some similarities, the growth properties of L- and H-GASC were significantly different from those obtained from normal tissues(82, 149, 183), and strictly resembled stem cells previously isolated from neoplastic livers(149).

Specifically, GASC possessed the ability to form transformation foci (data non shown) and to grow in an anchorage-independent fashion, when cultured in soft agar for 30 days (Figure 4.7 A-C).

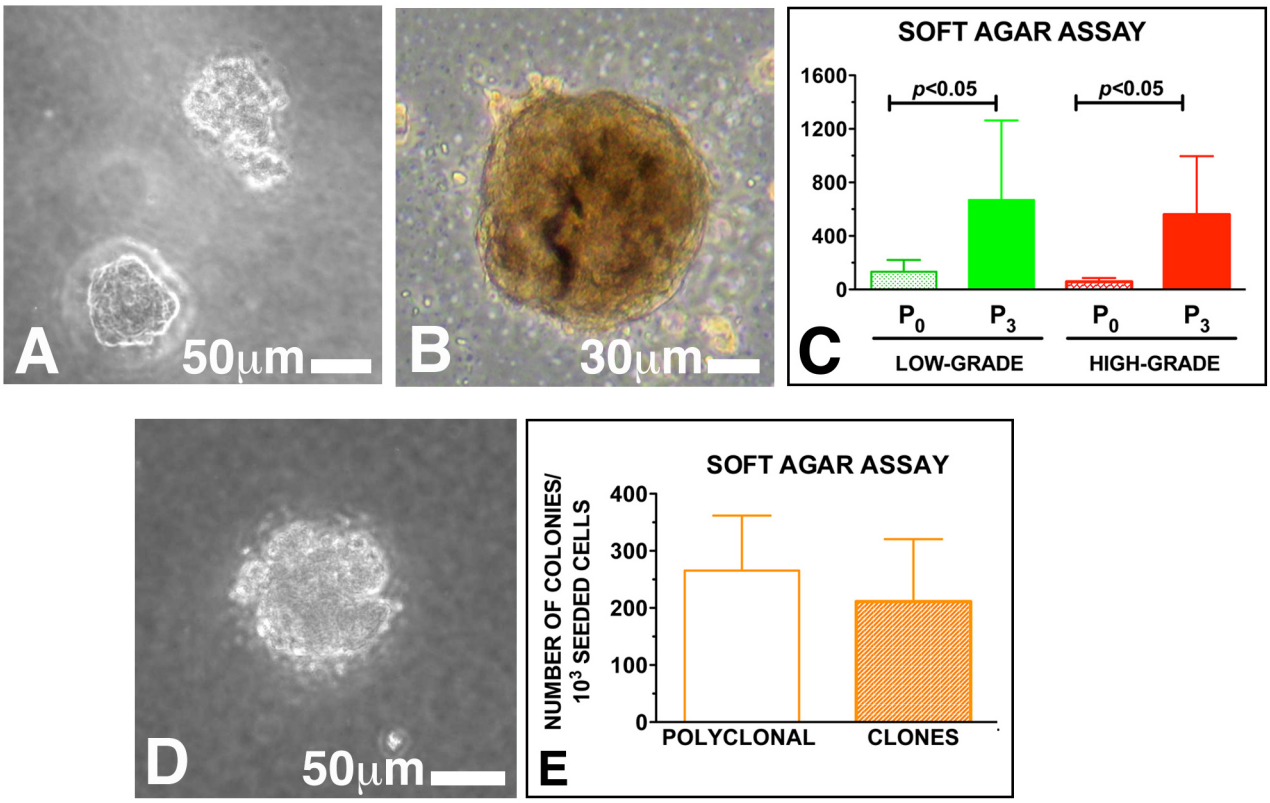
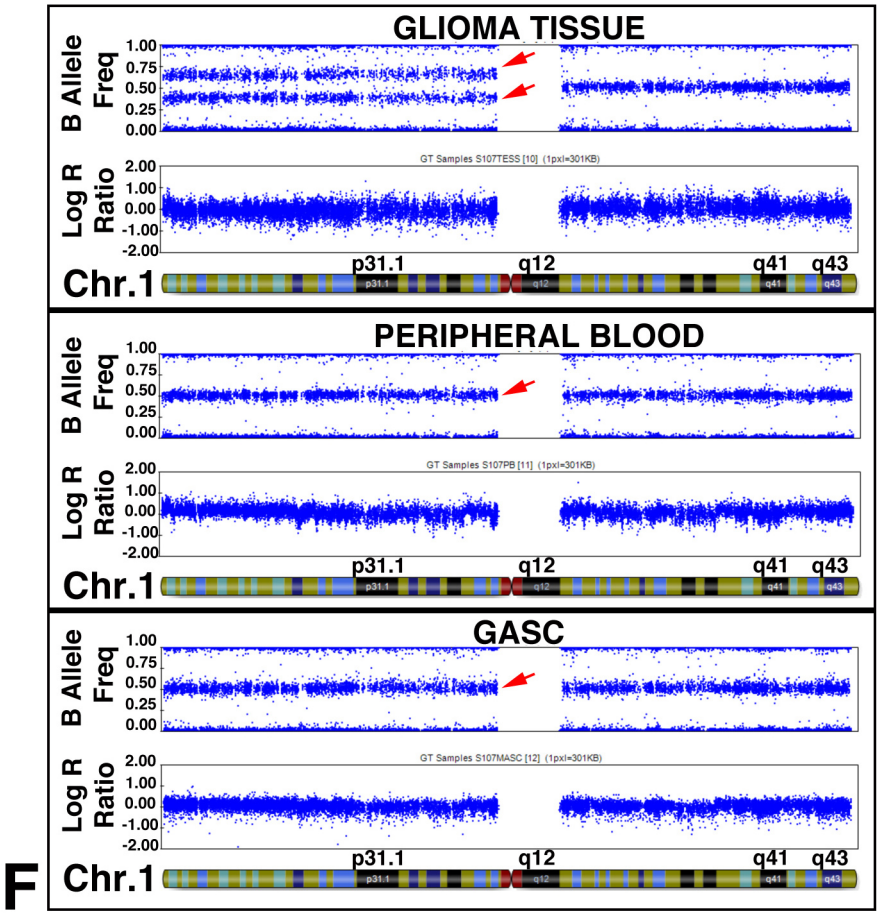


Figure 4. 7. (A-E) Anchorage-independent growth of GASC. Phase contrast images of colonies in soft agar of LGG- (A), HGG- (B) GASC. Single cell derived clones retain this property (D). (C, E) Results are expresses as mean ± standard deviation.

The ability to grow in semisolid medium is a property shared by tumorigenic cells and, in the solid tumor of epithelial origin, by tumor associated fibroblasts (189). Differences between H-GASC and L-GASC were statistically significant, being the fraction of cells able to grow in a semisolid medium significantly increased in cell lines obtained from high-grade gliomas (Figure 4.7 C). Importantly, with respect to isolated cells, the ability of growing in soft agar increased at the third passage in culture, demonstrating the ability of the culture conditions to maintain this aberrant growth property (Figure 4.7 C), and was retained by cells after single-cell cloning (Figure 4.7 D-E). Despite this aberrant growth property, L- and H-GASC differed from glioma-initiating cells because: they were growing in adhesion, they did not express CD133 and they were characterized by a mesenchymal stem cell phenotype. Moreover, when injected into the striatum of NOD-Scid mice (n=26), 10^5 either polyclonal (n=12) or clonal GASC (n=12) were unable to give rise, even after 8 months, to tumors, while B2C cell lines (n=2), used as control, did (data not shown).

In order to establish whether GASC cell lines presented, at a genetic level, the glioma mutations characterizing the respective tumor tissue, we performed a whole genome SNP analysis (n=4). Specifically, comparing the SNP profile of each GASC-line with the ones of the respective tumor of origin (positive control) and the one of the mononuclear cells isolated from the peripheral blood

(negative control), all the tested GASC lines were devoid of the genetic alterations present in the glioma tissues, while they shared the same SNP profile of the respective blood mononuclear cells



F

Figure 4. 7 (F) Representative SNP's analysis. While glioma tissue (upper panel) was characterized by a deletion in the chromosome 1 (1p), GASC (lower panel), as well as the mononuclear fraction of the peripheral (middle panel), obtained from the same patient, did not present the 1p deletion.

Similarly, when we tested, by FISH, GASC obtained from glioma samples characterized by the presence of 1p and/or 19q chromosme deletions (n= 15), we did not assess the presence of the deletions (Figure 4.7 G).

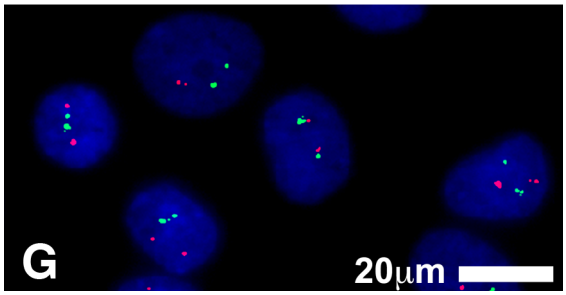


Figure 4. 7 G. FISH analysis performed on GASC did not reveal an imbalance in the number of spots corresponding to 1p36 (red fluorescence) and the number of spots corresponding to the 1q25 region of chromosome 1 (green fluorescence). Nuclei are depicted by the blue fluorescence of DAPI staining.

In conclusions, GASC although displaying aberrant growth properties, are devoid of genetic alterations and are not able to originate a tumor when injected in vivo. Interestingly, the GASC aberrant growth properties persist despite extensive culture suggesting some possible epigenetic mechanisms (128, 138).

4.4 GASC are characterized by a tumor supporting phenotype.

The aberrant growth properties displayed by GASC in the absence of genetic mutation strictly reminds the distinguishing features of tumor-associated fibroblasts (TAF) or cancer-associated fibroblasts (CAF)(128, 190). These latter cells reside in the tumor microenvironment of the epithelial tumors and are known to play a fundamental role in supporting growth, malignancy and metastatization of the tumor itself(128, 142, 190). It has been shown that human bone marrow–derived mesenchymal stem cells (hMSCs) exposed to tumor-conditioned medium (TCM) over a prolonged period of time assume a cancer associated fibroblast-like phenotype (141). Specifically, TCM-treated hMSCs were able to promote tumor cell growth both in vitro and in vivo and expressed myofibroblast markers, including a-smooth muscle actin, and fibroblast surface protein(141). More recently, we have shown that also mesenchymal stem cells resident in the liver can acquire an activated phenotype when exposed to a medium conditioned by hepatocellular carcinoma cell lines(149).

In order to establish whether L- and H-GASC possessed, in analogy to TAF, the ability to modify the biological properties of tumor cell lines, we cultured A-172 and U87 MG glioma cell line for three passages in a medium semi-conditioned by GASC (n=5). At the end of the treatment, A172 and U87 MG were assessed in terms of growth kinetics, migration ability and adhesion-independent growth.

With respect to cells grown in non-CM, both A172 and U87 MG grown in CM displayed a significant reduction of the population doubling time (Figure 4.8 A), and an increased capacity to grow in an anchorage-independent way, as demonstrated by soft agar assay (Figure 4.8 B),

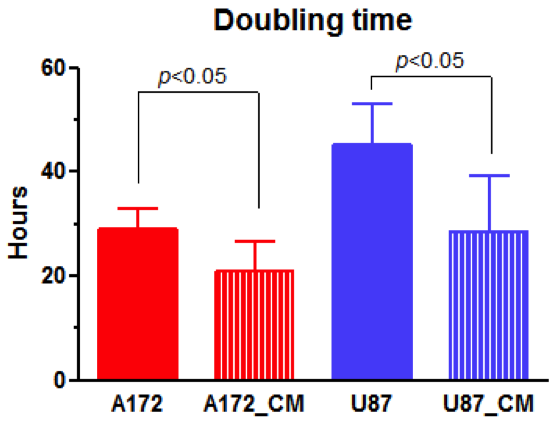


Figure 4.8.A Growth Kinetic. A172 and U87 growth in a medium conditioned by GASC (A172_CM and U87_CM) (bar)presented, with respect to the same lines growth in a non-conditioned medium, a significantly reduced population doubling time. Results are expressed as ± standard deviation.

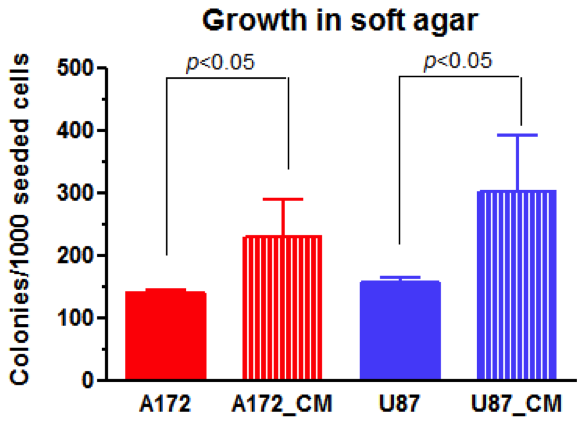


Figure 4.8.B Anchorage-indipendent growth. A172 and U87 growth in a medium conditioned by GASC (A172_CM and U87_CM) (bar) presented, with respect to the same lines growth in a non-conditioned medium, a significantly increased ability to grow iin soft agar. Results are expressed as ± standard deviation.

Interestingly, the growth in medium conditioned by GASC, significantly affected the migration ability of both A172 and U87 MG, as assessed by scratch assay, although in an opposite way (Figure 4.8 C). Specifically, A172 cultured in GASC-CM displayed, with respect to cells grown in non-CM, an 80% increased in the migration speed (15± 2 vs 27± 7µm/h, p=0.018); conversely, U87 MG cells cultured in GASC-CM decreased their migration speed of about 40% (32± 9 vs 19± 4µm/h, p=0.018)

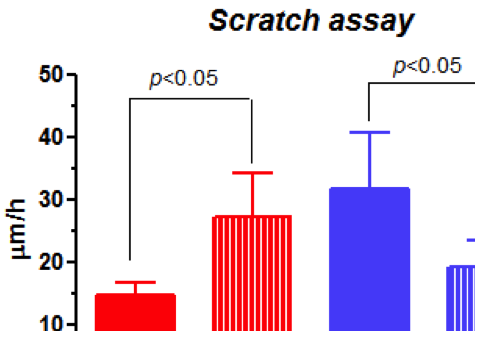


Figure 4.8.C Migration ability. A172 and U87 growth in a medium conditioned by GASC (A172_CM and U87_CM)(bar) presented, with respect to the same lines growth in a non-conditioned medium, a significantly, although in a opposite way, ability to migrate upon scratch. Results are expressed as ± standard deviation

With regard to the expression of markers that could be associated with an activated phenotype, we assessed that GASC displayed a protein expression pattern characteristic of reactive astrocytes. Specifically, a high fraction of the cells expressed vimentin, nestin, beta-3-tubulin and neuron specific enolase while GFAP was expressed in the 10% of the cells (Figures 4.5 and 4.6). Moreover, a large fraction of cells expressed VEGF and some cells expressed smooth muscle actin, both possible indicators of the myofibroblastic and pro-angiogenic potential of supporting cells (126, 138, 142). Quantitatively, no significant differences in the expression of these markers were detected between H- and L-GASC (data not shown).

Altogether, these results showed that GASC obtained from low- and high-grade gliomas are characterized, beside their stem cell properties, by aberrant growth properties and morphological and functional features that support their possible role as tumor supporting cells.

4.5 GASC features can predict LGG patient prognosis.

Since GASC showed a state of activation that did not change with the extensive culture, we wandered whether these cells could represent an *in vitro* model mimicking the biological properties of the patient tumor. To evaluate whether GASC could fulfill these expectations, we adopted a multi step approach (Figure 4.9);

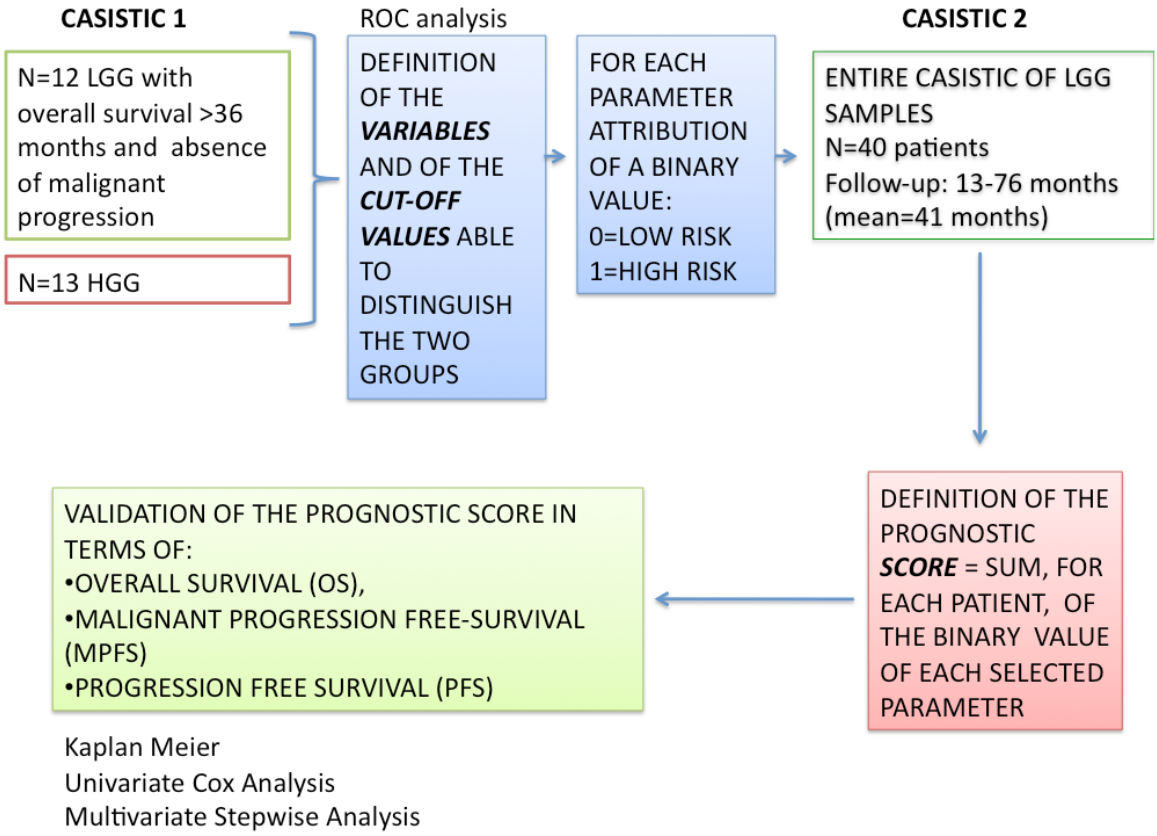


Figure 4.9. Definition and validation of the GASC-based score for the prognostic stratification of LGG patients. The picture recapitulates the workflow followed, the casistics used and the statistical tests adopted.

we first identified the GASC-features distinguishing LGG from HGG (Table 4.4) and we inserted them in a score that was finally tested for its capacity to prognostically stratify 40 LGG patients (Table 4.5) in terms of OS (overall survival), MPFS (malignant-progression free survival) and PFS (progression-free survival).

	LGG (N=12)	HGG (N=13)						
AGE, YEARS	33 (17-54)	63 (45-70)						
SEX, N(%) MALE	5 (42%)	9 (62%)	ROC AREA	95% CI**	P	CUTPOINT (%)	SENSITIVITY (%)	SPECIFICITY (%)
POPULATION DOUBLING TIME, HOURS	29 (24-47)	35 (22-65)	0.68	0.46-0.88	0.16			
CD133 %	0.26 (0.05-0.39)	1.1 (0.5-5.6)	1.00	0.86-1.00	<0.0001	**>0.5**	100	100
ABCG2 %	1.26 (0.14-1.69)	3.8 (0.5-11.2)	0.79	0.58-0.93	0.004	**>1.7**	69	91
CD49A %	96.8 (85.1-99.8)	77.2 (53.8-98.8)	0.90	0.74-0.99	<0.0001	**<84**	100	69
CD49D %	97 (39.5-99.2)	67.9 (19.9-97.2)	0.81	0.59-0.93	0.0008	**<88**	92	69
E-CADHERIN %	1.1 (0.3-22.13)	14.1 (0.6-77.5)	0.79	0.59-0.93	0.0021	**>6.3**	69	75
CD271 %	0.25 (0.02-0.80)	0.68 (0.24-4.78)	0.87	0.68-0.97	<0.0001	**>0.5**	75	83
CD44 %	99.5 (34.6-100)	93.2 (15.6-99.8)	0.75	0.52-0.90	0.0145			
CD34 %	0.24 (0.00-2.75)	0.77 (0.03-7.48)	0.59	0.37-0.78	0.18			
CD117 %	3.1 (0.2-78.8)	1.7 (0.2-65.9)	0.57	0.35-0.76	0.55			
CD29 %	89.5 (43.4-99.7)	70.8 (1.4-95.9)	0.74	0.55-0.91	0.017			
N-CADHERIN %	1.5 (0.0-57.0)	2.0 (0.0-33.8)	0.55	0.34-0.77	0.72			
CD13 %	99.9 (98.8-100.0)	99.6 (96.6-100.0)	0.67	0.47-0.87	0.147			
CD59 %	99.8 (99.2-99.9)	99.8 (97.4-100.0)	0.58	0.36-0.79	0.516			
CD105 %	99.7 (40.6-100.0)	32.3 (0.9-99.9)	0.85	0.63-0.95	<0.0001	**<38**	100	54
CD90 %	77.0 (26.5-92.4)	98.2 (79.8-99.4)	0.94	0.74-0.99	<0.0001	**>90**	85	92
CD73 %	99.9 (97.7-100.0)	99.1 (91.1-99.9)	0.85	0.66-0.97	<0.0001	**<99.5**	91	58
CD10 %	67.4 (0.2-85.8)	19.2 (0.4-76.3)	0.62	0.35-0.85	0.467			
CD66E %	0.36 (0.17-0.53)	7.2 (0.1-86.9)	0.68	0.45-0.86	0.179			

Table 4.4. Definition of the parameters to include in the GASC-related score. ROC analysis and cut-off value of the GASC-based parameters differentiating HGG from LGG*.

*** LGG characterized by an OS ≥36 months and without RNM evidence of malignant transformation.**

**** Exact binomial estimation of confidence intervals (De Long).**

CLINICOPATHOLOGIC FEATURES	PATIENTS, NO.	%	MEDIAN (RANGE)
SEX			
MALE	25	62.5	-
FEMALE	15	37.5	-
AGE AT SURGERY (YEARS)	-	-	38.5 (18-63)
EXTENT OF RESECTION (%)	-	-	88 (25 - 100)
TUMOR SUBTYPE			
ASTROCYTOMA	26	65	-
OLIGOASTROCYTOMA+ OLIGODENDROGLIOMA	14	35	-
KI67 EXPRESSION (%)			5 (1 – 20)
≤ 4	18	45	-
>4	22	55	-
P53 EXPRESSION (N=37)	29	78	-
IDH1 MUTATION (N=35)	28	80	-
IDH2 MUTATION (N=35)	2	5.7	-
IDH1 OR IDH2 MUTATION (N=35)	30	85	-
CHROMOSOME 1P DELETION (N=30)	10	33	-
CHROMOSOME 19Q DELETION (N=30)	13	43	-
CHROMOSOME 1P AND 19Q CO-DELETION (N=30)	10	33	-
MGMT PROMOTER METHYLATION (N=31)	26	84	-
NUMBER OF MITOSIS/10HPF	-	-	1 (0 - 7)
POST-OPERATIVE CHEMOTHERAPY	15	37.5	-
POST-OPERATIVE RADIOTHERAPY	23	57.5	-
2ND SURGERY	10	25	-

Table 4.5 clinicopathologic feature of the n=40 low grade-glioma patients

4.5.1 Definition of the GASC-based score

Starting from a case study composed of 13 H-GASC and 12 L-GASC, obtained, these latter, from patients with an OS>36 months and without MRI evidence of malignant progression, and utilizing a ROC analysis, we selected 9 parameters significantly ($p<0.005$) able to correctly classify the two groups and we determined the cut-off value able to discriminate the two populations (Table 4.4).

Of the 9 parameters, 5 resulted to be more expressed (CD133, CD271, ABCG2, E-Cadherin, CD90) and 4 less expressed (CD49a, CD49d, CD105, CD73) in H-GASC with respect to the L- GASC, and we expressed the selected parameters as a binary value (Figure 4.10A).

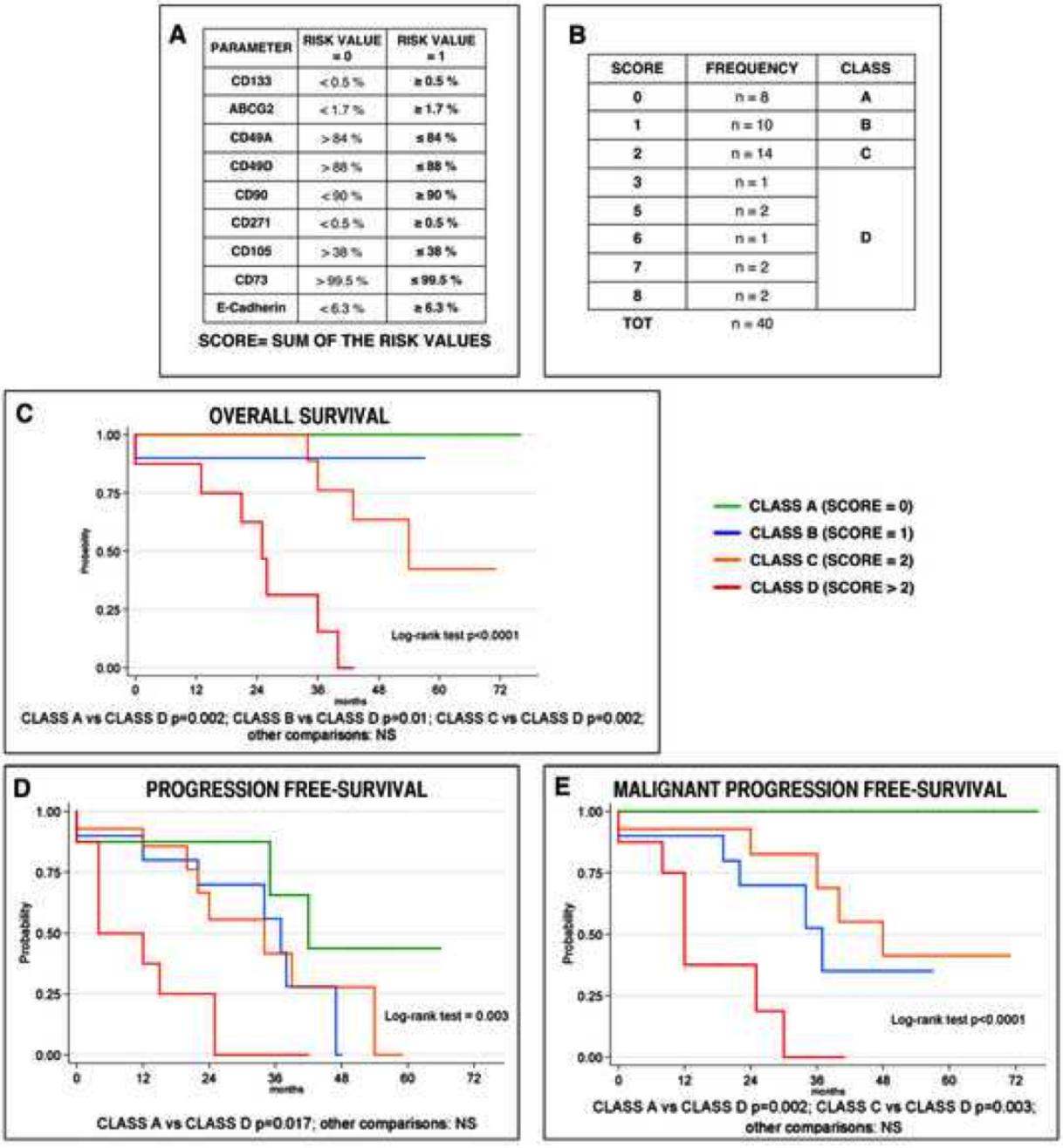


Figure 4.10. Prognostic value of the GASC-based score in LGG patients. (A) Parameters and cut-off value used to define the GASC-related score. (B) Distribution of the LGG patients in GASC-classes, according to the GASC-score value. (C-E) Kaplan-Meier curves showing OS (C), PFS (D) and MPFS (E) in LGG patients stratified according to the GASC-baesd score.

Finally, we created a score based on the sum of the 9 binary values (Figure 4.10A) and we assessed the prognostic value of the determined parameter in a larger casistic including 40 subsequent LGG-patients (median follow-up 36 months, range 13-76) (Table 4.6).

As illustrated in Figure 4.10B, 8 of the LGG patients presented a score=0, 10 a score= 1, 14 a score=2, 1 a score=3, 2 a score=5, 1 a score=6, 2 a score=7 and 2 a score=8. We decided, then, to stratify the score in the following 4 classes: A (score=0), B (score=1), C (score=2) and D (score>2).

4.5.2 Evaluation of the prognostic value of the GASC-based score

4.5.2.1 Clinicopathologic features and prognostic-predictive biomarkers of LGG

Table 4.5 summarizes the demographic, clinical and histological data of the 40 LGG patients analyzed. As shown, LGG were analyzed in terms of tumor histological subtype, mitotic index, p53 and Ki67 expression, presence of IDH1 and IDH2 mutations, chromosome 1p- and/or chromosome 19q-deletion and MGMT promoter methylation. *Overall survival (OS)*

Overall there were 12 deaths (30%) and the median follow-up in the surviving patients was 41 months (range 19-76 months). The estimated 5-year OS rates were 100% (score=0), 90% (score=1), 42% (score=2) and 0% (score>2), respectively (log-rank test: $p<0.0001$, Figure 4.10C).

Table 4.6 summarizes the prognostic factors associated with OS at univariate analysis ($p<0.05$). Specifically, the extent of tumor resection (EOR), treated as continue variable, was associated with a significant improvement in OS (HR 0.97, 95%CI 0.94- 0.99). Even higher protective role was played by the presence of mutated IDH1 or IDH2 genes (HR 0.11, 95% CI 0.03-0.40). Conversely, the OS was significantly poorer in older patients (HR 1.05, CI 95% 1.00-1.09). Importantly, the GASC-based score was associated with a worse OS, either when treated in its original form (HR 1.85, CI 95% 1.41-2.44) or in four classes (HR 6.52, CI 95% 2.26-18.86).

No significant prognostic roles were played by other histological parameters, including Ki67 expression (Ki67≤4 and Ki67>4; p=0.516). Finally, the multivariate Cox analysis showed that the four classes GASC-based score was the only independent predictor of OS (HR 8.84, CI 95% 2.15-36.28, p=0.002).

Covariates	OS			PFS			MPFS		
	HR	**95% CI**	**P**	**HR**	**95% CI**	**P**	**HR**	**95% CI**	**P**
AGE (MODELED AS CONTINUOUS VARIABLE)	1.05	1.00-1.09	**0.032**	1.02	0.99-1.052	0.182	1.04	1.00-1.08	**0.038**
GENDER (FEMALE VS MALE)	0.41	0.11-1.51	0.180	0.83	0.36-1.91	0.663	0.55	0.19-1.57	0.265
% EOR (MODELED AS CONTINUOUS VARIABLE)	0.97	0.94-0.99	**0.015**	0.96	0.94-0.98	**<0.0001**	0.97	0.95-1.0	**0.022**
TUMOR SUBTYPE OLIGOASTROCYTOMA/ OLIGODENDROGLIOMA VS FIBRILLAR ASTROCYTOMA	1.35	0.43-4.26	0.610	0.74	0.31-1.80	0.511	0.81	0.28-2.30	0.688
% KI67 > 4 VS ≤ 4	1.49	0.45-4.99	0.516	2.05	0.86-4.86	0.104	2.01	0.70-5.73	0.193
NUMBER OF MITOSIS (MODELED AS CONTINUOUS VARIABLE)	1.40	0.93-2.11	0.111	1.22	0.88-1.67	0.229	1.37	0.99-1.91	0.061
IDH1 MUTATION YES VS NO	0.13	0.04-0.47	**0.002**	0.33	0.12-0.86	**0.024**	0.24	0.08-0.68	**0.007**
IDH2 MUTATION YES VS NO	1.73	0.22-13.72	0.603	0.58	0.08-4.37	0.595	0.95	0.12-7.26	0.958
IDH1 OR IDH2	0.11	0.03-0.40	**0.001**	0.09	0.02-0.31	**<0.0001**	0.12	0.04-0.40	**0.001**

MUTATION YES OR NO									
P53 EXPRESSION YES VS NO	0.59	0.17-2.04	0.408	1.25	0.42-3.74	0.691	0.86	0.27-2.70	0.795
MGMT PROMOTER METHYLATION YES VS NO	0.40	0.07-2.21	0.296	0.43	0.13-1.36	0.149	0.21	0.06-0.80	**0.022**
CHROMOSOME 1P-DELETION YES VS NO	0.37	0.08-1.73	0.205	0.33	0.11-1.04	0.058	0.25	0.054-1.13	0.072
CHROMOSOME 19Q-DELETION YES VS NO	0.24	0.05-1.18	0.080	0.35	0.12-1.00	**0.050**	0.16	0.03-0.73	**0.018**
CHROMOSOME 1P/19Q CO-DELETION YES VS NO	0.37	0.08-1.73	0.205	0.33	0.11-1.04	0.058	0.25	0.054-1.13	0.072
GASC-BASED SCORE (MODELED AS CONTINUOUS VARIABLE)	1.85	1.41-2.44	**<0.0001**	1.52	1.25-1.87	**<0.0001**	1.58	1.30-1.93	**<0.0001**
GASC-BASED SCORE (MODELED AS CATEGORIC VARIABLE)	6.52	2.26-18.86	**0.001**	1.83	1.18-2.82	**0.007**	2.96	1.57-5.60	**0.001**

Table 4.6. Univariate analysis of clinical, histological and GASC-based parameters with Overall Survival (OS), Progression-Free Survival (PFS) and Malignant Progression-Free Survival (MPFS) in 40 patients with Low-Grade-Gliomas.

4.5.2.2 Tumor Progression and Malignant Transformation

Tumor progression was identified in 25 (62.5%) cases, while malignant progression (new contrast enhancement on follow-up imaging) was observed in 17 (42.5%) cases. A total of 5 of the 17 patients with malignant progression underwent a second surgery. In all cases a higher-grade tumor was identified. Among the remaining 12 cases, 7 underwent chemo- and radio-therapy, 3 radiotherapy only and 1 chemotherapy only. The estimated 2-year and 5-years PFS rates were

87.5% and 43.7% (score=0), 80% and 0.0% (score=1), 66.7% and 0.0% (score=2) and 25% and 0% (score>2), respectively (log-rank test: p=0.003, Figure 4.10D). Table 4.6 summarizes the prognostic factors associated with PFS at univariate analysis. Specifically, the EOR, treated as continue variable, was associated with a significant improvement in PFS (HR 0.96, CI 95% 0.94-0.98). Higher protective role was played by the presence of mutated IDH1 or IDH2 genes (HR 0.09, CI 95% 0.02-0.31). Again, the GASC-based score was associated with a worse PFS, either when treated in its original form (HR 1.52, CI 95% 1.25-1.87) or in four classes (HR 1.83, CI 95% 1.18-2.82). No significant prognostic roles were played by other clinical and histological parameters. Finally, the multivariate Cox analysis showed that the only independent predictor of PFS was EOR (HR 0.96, CI 95% 0.93-0.98, p=0.003).

Considering the MPFS in the 4 GASC-based score classes, the estimated 2-year and 5- years MPFS rates were 100% and 100% (score=0), 80% and 35% (score=1), 92.9% and 41.3% (score=2) and 37.5% and 0% (score>2), respectively (log-rank test: p<0.0001, Figure 4.10E).

The prognostic factors associated with MPFS at univariate analysis are summarized in Table 4.6. Specifically, the EOR was associated with a significant improvement in MPFS (HR 0.97, CI 95% 0.95-1.0). Even higher protective role was played by the presence of mutated IDH1 or IDH2 genes (HR 0.12, CI 95% 0.04-0.40) and methylation of the MGMT-promoter (HR 0.21, CI 95% 0.06-0.80). Conversely, the MPFS was significantly poorer in older patients (HR 1.04, CI 95% 1.00-1.08). Importantly, the GASC-based score was associated with a worse MPFS, either when treated in its original form (HR 1.58, CI 95% 1.30-1.93) or in four classes (HR 2.96, CI 95% 1.57-5.60). No significant prognostic roles were played by other clinical and histological parameters.

Again, the multivariate Cox analysis showed that the only independent predictor of MPFS was the four classes GASC-based score (HR 3.74, CI 95% 1.60-8.75, p=0.002). Altogether, these results indicate that the GASC-based score is significantly associated with LGG-OS, -MPFS and -PFS and represents, among the state-of the art clinical, histological and molecular LGG prognostic factors, the strongest independent predictor of OS and MPFS.

5 DISCUSSION

The purpose of personalized medicine (as recognized by NIH and FDA) is to allow clinicians to identify patient-specific and tumor-specific factors that can be used to select maximally effective therapies and minimize treatment-related toxicity.

This is crucial in neuro-oncology, given the morbidity and mortality of brain tumors, and the rarity of effective treatment options coupled with the frequent development of resistance and/or potential toxicity of therapy. In fact, for low-grade gliomas (LGG) there are not definitive criteria to classify a lesion as at high- or low- risk to progress and novel drugs specifically designed to target LGG are not yet available. High-grade gliomas (HGG) are characterized by an extremely severe prognosis (although rare long-term survivors have been reported) and current therapies fails in significantly affect overall survival, often because of resistance.

In the recent years, some advances in defining novel biomarkers able to better classify both LGG and HGG have been done(95).

Regarding LGG, IDH mutations and 1p/19q co-deletions seem to be largely independent prognostic markers for overall survival, given a genotoxic treatment is provided(26), while no known biomarker is of any relevance for the postoperative course of disease in the absence of a genotoxic treatment(26). BRAF aberrations have the potential of assuming a predictive biomarker function in pilocytic astrocytoma, given a BRAF inhibitor-based treatment is applied(26).

Considering HGG, IDH1 mutations are considered a good marker in identifying secondary glioblastoma(40), while genetic loss on chromosomes 1p/19q (LOH 1p/19q) is considered as a marker for responsiveness to chemotherapy and it describes a distinct tumour entity (oligodendroglioma) with a prolonged natural history irrespective of treatment(37). In retrospective analyses the epigenetic silencing of the methyl-guanine methyl transferase (MGMT) gene promoter by methylation has been correlated with outcome to alkylating agent chemotherapy(37). Conversely, the prognostic/predictive role of p53 and Ki67 expression has been questioned.

In carcinomas, Researches are currently exploiting novel biomarkers studying the tumor-associated fibroblasts (TAF) and infiltrating mesenchymal stem cells (MSC). In fact, *tumor-associated cells* seem to play an active role in tumorigenesis, therefore, the possibility to create an in vitro model representative of the tumor stroma can represent a key element for a patient-based approach aimed at identifying novel prognostic and predictive markers as well as novel therapeutic targets (191).

TAF with macrophages, lymphocytes, endothelial cells and pericytes, constitute the tumor microenvironment that interacts and influence tumor progression through the release of growth factors, proteases and cytokines (126, 128, 138). Regarding the possible origin of these stromal cells (139, 142), they may result from the recruitment of resident tissue stem cells, or may be the result of epithelial to mesenchymal transition of tumor parenchymal cells or may arise from the recruitment of bone marrow-derived cells from the circulation (192). In fact, several experimental evidences have suggested the possibility that mesenchymal stem cells derived from bone marrow may acquire the properties of TAF, when exposed to a medium conditioned by several neoplastic cell-lines(85, 142).

In our laboratory we have optimized a methods to isolate multipotent adult stem cells from healthy and neoplastic human tissues (82, 149, 183). These cells named MASC were characterized by a mesenchymal immunophenotype, expression of pluripotent state trascription factors and were characterized also by a wide differentiation capacity. Applying the same method to ovarian adenocarcinomas and hepatocellular carcinomas(149), it was demonstrated, in addition to their characteristics of stemness, that the MASC isolated from neoplastic epithelial tissues, showed, *in vitro*, some of the phenotypic characteristics and typical functional properties of TAF.

We therefore decided to apply the same method for growing cells from LGG- and HGG- samples.

Compared to MASC obtained from non-neoplastic tissues, the cells isolated from LGG and particularly those isolated from HGG, were characterized by a significantly reduction in the colony forming efficiency. This parameter is often considered an index of the frequency of the analyzed population in the most primitive cell suspension. This could assume that gliomas are characterized by a lower frequency of these cells. Another explanation may be linked to the presence, especially in HGG, of necrotic area and hemorrhagic lesions, which can determinate, particularly in primary cultures, the release of factors capable of interfering with cell growth (R. Ian Freshney. Basic Principles of Cell Culture. In: Culture of Cells for Tissue Engineering, edited by Gordana Vunjak-Novakovic and R. Ian Freshney Copyright 2006 John Wiley & Sons, Inc.).

In any case, the high efficiency of the isolation method and the growth kinetics of the cells, even when starting from a small amount of material, allows obtaining large amount of cells (million of cells) in a relatively short time (2-3 weeks). Therefore, this methods could be used to get a number of cells adequate for high-throughput studies; moreover, it makes virtually possible to set up cell cultures that can be used for assays aimed at defining prognosis or drug sensitivity/resistance for the same specific patient from which cells were obtained.

When analyzed at the third passage in culture, LGG-derived cells (named L-GASC) and HGG-derived cells (named H-GASC) were characterized by a mesenchymal surface immunophenotype, similar to the one of MASC isolated from normal human tissues such as liver, bone and heart (82).

To confirm that GASC were characterized by stem cell properties, we first evaluated the expression of pluripotent-state specific transcription factors, assessing that Oct-4, Nanog and Sox-2 were expressed both at mRNA and protein level. Moreover, GASC were characterized by clonogenicity and multipotency, being able to differentiate into neuronal, glial and oligodendroglial derivatives, even when cloned at single cell level.

Beside their stem cell features, GASC were characterized by aberrant growth properties. In fact, L- and H-GASC were able to form colonies when grown in semi-solid medium. This property characterizes not only tumorigenic cells but also TAF. In fact, San Francesco and collaborators in 2004 showed that CAF demonstrated a much greater potential to undergo anchorage independent growth, compared to Normal Human Prostate Fibroblast. Expression of TGF-1 was higher in CAF than in NHPF, which may account for the ability CAF to form colonies in soft agar and promote prostate epithelial tumor progression (189).

To verify if GASC, similarly to what have already been described in the literature (85, 142), possess the ability to modify the biological behavior of tumor cells *in vitro,* we cultured two commercial glioblastoma cell lines, A172 and U87, with o without a medium conditioned by GASC. When compared in terms of growth kinetic and anchorage-independent growth, A172 and U87 grown in GASC-conditioned medium were characterized by a significant increase in both growth speed and ability to grow in an anchorage-independent way. Conversely, the GASC-conditoned medium determined a significant but opposite different effect on cell migration. In fact, while U87 in the GASC-conditioned medium decreased by 40% their speed in closing the scratch, A172 increased their speed by 80%. These data are in agreement with what is present in the literature on the possible dual ruole of TAF on tumor growth (132, 155). In fact TAF, depending on the context, can either promote or inhibit the neoplastic progression (132, 155).

To our knowledge, this is the first time that it has demonstrated the possibility to isolate and expand from human glioma samples a population of primitive cells with characteristic of stromal supporting cells.

The presence, in gliomas, of “mesenchymal" stem cells with an "activated phenotype” raises issues regarding the origin of these cells and their mechanisms of activation.

Concerning the origin of these cells, it is important to note that TAF have been described in the context of epithelial neoplasms, demonstrating their analogy to the fibroblasts usually present in the context of wound healing process (126, 128, 138). For this reason, these cells are named tumor-

associated fibroblasts (TAF). GASC shared some immunophenotypic and functional similarities with TAF. However, the presence of fibroblasts in the central nervous system is limited and it has as not been described their proliferation in the course of pathological processes (193). Conversely, the reactive processes of the CNS are characterized by the presence of a population of reactive astrocytes that are characterized by a decreased expression of GFAP and by an increased expression of vimentin, nestin, Tubulin beta-3 and neuron-specific enolase (194-196) suggesting a possible origin of GASC from these cells. Interestingly, xenotransplantation experiments employing human glioma cell lines have suggested that astrocytes in the vicinity of glioma cells can be activated and facilitate tumor invasiveness (158). In addition, genetic fate mapping studies have shown that reactive glia could acquire a stem cell potential outside the two major neural stem cell niches in adult mammalians (178, 179). Similarly, Platelet Derived Growth Factor (PDGF)-induced gliomas arising in both adult and neonatal rats have been shown to contain normal stem cells and progenitor cells ‘‘recruited’’ into the glioma mass and induced to proliferate, supporting the hypothesis that proliferative stem-like portions of the tumor can arise from normal progenitors (180). Therefore, it would be very interesting to prospectively isolate, in humans, the cell population able to give rise to GASC in vitro. These could help verifying their location, their nature and whether or not they could derive from mesenchymal cells (either resident or recruited to the site of damage), or from other glial cells.

Regarding the mechanisms that lead to a state of permanent activation, the absence, in GASC, of the chromosomal aberrations present in the original tumor, not only confirmed the non-neoplastic origin of the cell lines, but it has placed emphasis on possible epigenetic mechanisms, whose definition may provide new information on the biology of both TAF and tumor cells (126, 128, 138).

We finally wondered if the study of GASC could have a possible clinical significance. It is important to remember, as described above, that novel prognostic and predictive markers able to better guide the clinical practice are required both in the LGG- and HGG- context. In the LGG, clinical, histopatological and neuroradiological features fail in defining the fraction of patients characterized by a faster progression and a possible rapid transformation into a malignant phenotype. These patients may benefit from adjuvant treatments, whose sides’ effect would not be acceptable in patients with a long disease-free survival.

Since GASC cultures are characterized by an activated phenotype, that is stable with passages, we hypothesized that it could represent an *in vitro* model of the stromal component supporting the tumor growth *in vivo*. As mentioned, we compared the features of GASC obtained from HGG vs long-survivor LGG. Interestingly, among the analyzed GASC parameters (population doubling

time, ability to grow in soft agar, expression of the surface markers CD44, CD34, CD117, CD133, ABCG2, CD29, CD49A, CD49d, N-CAD, E-CAD, CD13, CD59, CD105, CD90, CD73, CD271, CD10, CD66e, CD45, HLA-DR, CXCR4, CD38, N-CAD and KDR and expression of the cytoplasmic proteins nestin, vimentin, tubulin beta-3 and neuron-specific enolase), we identified 9 surface proteins (CD133, ABCG2, CD49a, CD49d, CD73, CD90, CD105, E-cadherin and CD271), enabling us to discriminate HGG and LGG.

Importantly, when these cell-based score was analyzed in a multivariate analysis comprehending the commonly considered prognostic factors (Ki67 expression, IDH1 mutation, 1p and 19q deletion, age of patient and extent of resection), it resulted to be an independent predictor of both OS and MPFS. These preliminary and promising data support the notion that the variability in the GASC cell lines obtained from patients with high and low-grade gliomas seems to reflect the biological and clinical variability of the tumors.

In conclusion:

1. It is possible to isolate, from both LGG and HGG, a population of multipotent stem cells that present immunophenotypic and functional characteristics of stromal supporting cells. These multipotent cells: 1. Can be easily propagated in culture, 2. Maintain, in culture, an activated phenotype and, therefore, 3. Represent a possible tool for understanding mechanisms and biology of gliomas;
2. Some of the features presented by GASC seem to correlate with OS and MPFS in LGG patient's, thus supporting the notion that GASC can represent a patient-based *in vitro* culture model representative of the human tumor.

The development of an *in vitro* culture model representative of the human tumor would offer the unique opportunity to study the biological features of the tumor and to hypothesized novel diagnostic, prognostic and predictive markers. Importantly, it would help in defining novel therapeutic strategies aimed at blocking the activation of the cells of the tumor microenvironment and their communication with the cancer cells (126, 128, 138). Moreover, this patient-based in vitro model would allow drug-screening strategies and it is prerequisite for developing novel in vivo models strictly resembling the tumor in situ.

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