UNIVERSITÀ DEGLI STUDI DI UDINE

CORSO DI DOTTORATO DI RICERCA IN SCIENZE E TECNOLOGIE CLINICHE CICLO XXVII

TESI DI DOTTORATO DI RICERCA

EXOSOMES RELEASED BY GLIOBLASTOMA STEM CELLS ACTIVATE ASTROCYTES AND INDUCE THE ACQUISITION OF A NEURAL STEM CELL POTENTIAL BY GLIAL CELLS

RELATORE:

Prof. Giacinto SCOLES

DOTTORANDO:

Dott. Damiano Mangoni

CORRELATORE:

Prof. Carlo Alberto Beltrami

CORRELATORE:

Dott.ssa Daniela Cesselli

ANNO ACCADEMICO 2014

INDEX

ABSTRACT

Cellular heterogeneity and plasticity is a hallmark of glioblastoma multiforme and the role played by the surrounding microenvironment is considered to be crucial for the tumor progression. Recent findings suggest that the glioblastoma microenvironment is populated by astrocytes that undergo a process of activation acquiring properties characterizing reactive gliosis, along with the acquisition or re-activation of a stem cell potential. However, communication processes occurring among the different cell types populating the glioblastoma microenvironment are largely unclear. We evaluated the possibility that exosomes produced by glioblastoma cells could modulate the biological properties of normal astrocytes inducing an activated phenotype and stem-like features.

The results obtained indicate that glioblastoma cells, both derived from a commercial cell line or primary cultured cells, are capable of inducing the activation of astrocytes by means of exosomes release. Indeed, after being grown in the presence of these latter, astrocytes acquire morphological, functional and phenotypic features distinguishing the phenotype of reactive astrocyte. However, only exosomes released by glioblastoma stem cells (GSC) promote the de-differentiation of normal human astrocytes, increasing their growth efficiency as spherical aggregates in suspension. These neurospheres, here called “astrospheres”, possess neural stem cell-like features, being able to express neural stem cell markers, and to differentiate into neuronal, glial and oligodendroglial lineages. Moreover, “astrospheres” obtained from astrocytes grown in culture in the presence of exosomes released by glioblastoma stem cells were able to spontaneously differentiate into functional radial glia-like cells, characterized by the expression of both neuroepithelial and astroglial markers and by the ability to support the migration of immature neurons, thus suggesting a possible mechanism through which glioblastoma cells infiltrate the brain parenchyma.

LIST OF ABBREVIATIONS

ASTRO/A: Astrocytes

AM: Astrocyte Medium

CSC: Cancer Stem Cells

EXO: Exosomes

GBM: Primary cultured glioblastoma cells

GSC: Primary cultured glioblastoma stem cells

LGG: Low-Grade Gliomas

HGG: High-Grade Gliomas

MVs: Microvesicles

NSC: Neural Stem Cells

RGC: Radial Glial Cells

TAF: Tumor-Associated Fibroblasts

1. 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).

Glial cells have many functions. They provide mechanical support to neurons and, because of their non-conducting nature, act as insulators between neurons and prevent neuronal impulses from spreading in unwanted directions. They can repair damaged areas of the nervous tissue by proliferation (gliosis), as they form a glial scar tissue by filling the gaps left by degenerated neurons, and they can remove foreign material and cell debris by phagocytosis. Glial cells help in neuronal functions by maintaining a suitable metabolic and ionic environment for neurons, and can take up and store neurotransmitters released by neighboring synapses.

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

- **Microglia**, of mesodermal origin;
- **Macroglia**, of ectodermal origin.

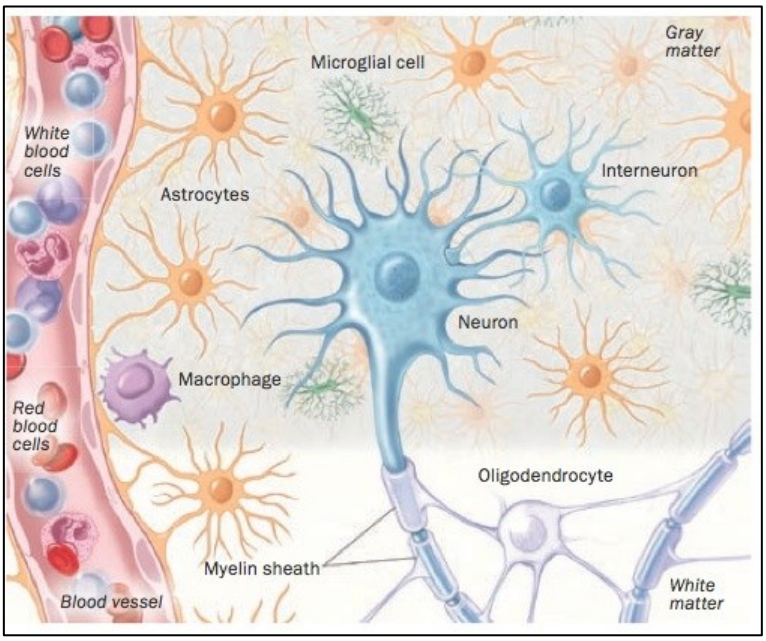

Figure 1.1. Glial cells of the CNS. From: http://www.jaynejubb.com/november2011article.htm

Microglia. These are the smallest of glial cells and are characterized by a flattened cell body with a few short, fine processes. They are often associated with capillaries and are considered phagocytic in nature. Microglial cells are possibly derived from 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 (1), and the two major views affirm that they derive either from neuroepithelial cells (2, 3) or from hematopoietic cells (4, 5).

Macroglia. It's mainly composed by astroglia and oligodendroglia, and it is generally considered developmentally distinct from microglia, being derived from neuroectoderm (6).

Astrocytes

Astrocytes have been divided in two main subtypes, *protoplasmic* and *fibrous*, on the basis of differences of their cellular morphology and anatomy location (7):

Protoplasmic astrocytes are found throughout all gray matter, and as first demonstrated using classical silver impregnation techniques, exhibit the morphology of several stem branches that give rise to many finely branched 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 astrocytes subtypes make extensive contacts with blood vessels.

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 (8). The oligodendrocyte is mainly a myelin-forming cell, but there are also satellite oligodendrocytes that may not be directly connected to the myelin sheath (9). Satellite oligodendrocytes are perineuronal and may serve to regulate the microenvironment around neurons (10, 11). A number of features consistently distinguish oligodendrocytes from astrocytes (11), 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 (12). 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 (11, 13).

Glioblast

Glioblasts are stem cells able to differentiate into macroglial cells. They are particularly numerous beneath the ependyma (14).

Ependymal cells

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 (15).

1.2 GLIOMAS

As previously mentioned, gliomas, which comprise astrocytic, oligodendrocytic 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 (16, 17). The most frequent (65%) and malignant histological type is the *glioblastoma multiforme* (16, 17). 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 Asians populations, but to some extent, this may reflect socio-economic differences and under-ascertainment in some regions, rather a significant difference in genetic susceptibility.

In Italy, 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 (16, 17). 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 (16, 17).

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. Approximatively 60% of mutations are located in the hot spot codons 248 and 273 and the majority of these are G:C-->AT 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 heterozygosity (LOH) of chromosome 19 has been described in anaplastic astrocytomas (16, 17).

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 (16, 17).

Today, the only environmental factor unequivocally associated with an increased risk of brain tumors is the 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 tumors, often within 10 years of therapy.

Other studies, although not conclusive, suggest cerebral traumas, nitrosamine rich food ingestion ad electromagnetic fields exposure (cell phones predominantly) as possible predisposing factors (16, 17).

The association between occupations carcinogens and glioma occurrence has been object of different studies, demonstrating an elevated risk of astrocytomas 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, tetrachloroethylene, trichloroethylene, but mostly chloride methylene (16, 17).

1.2.2. Glioma classification

The WHO classification, which incorporates the criteria of the St.Anne/Mayo, is usually recommended as a recent and updated international standard for classifying and grading gliomas (Table 1.1) (18).

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

Therefore, according to WHO, gliomas are:

- Divided on the basis of morphological 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 grade is based on histological criteria such as cellularity, nuclear atypia, vascularization and necrosis.

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

	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 tumors of the CNS. From (18)

On the basis of location, gliomas can be classified, according to whether they are above or below the tentorium (19, 20), a membrane of the brain, which separates the cerebrum from the cerebellum, in:

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

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

From a clinical point of view, gliomas are further categorized, depending on the biological aggressiveness, in (21):

- **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 worst prognosis.

The 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 tumors (22).

1.2.3. Low-Grade Gliomas

The term Low-Grade Gliomas (LGG) includes all WHO grade I and II gliomas (23). LGG categories include subependymal giant cell astrocytoma, pilocytic astrocytoma, pilomyxoid astrocytoma, diffuse astrocytoma, pleomorphic xanthoastrocytoma, oligodendroglioma, oligoastrocytoma and certain ependymomas (24). LGG are most common among men and white people at typically affect patients at a younger age than high-grade gliomas (fourth versus sixth decade of life). LGG most commonly involve the cerebral hemisphere, 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 (25). 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 (26). 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 neurocognitive function impairment, particularly radiotherapy; these side effects are not justifiable in patients that are possibly at low rick of relapse/progression; 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 (26-31), 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 (astrocytomas) (22, 32, 33). The IDH1 mutation is inversely correlated with grade, tightly associated with a 1p19q co-deleted genotype and a MGMT methylated status by mutually exclusive with Epidermal Growth Factor Receptor (EGFR) amplification and loss of chromosome 10 (32, 33). Moreover, abnormalities in the PTEN tumor-suppressor gene and the BRAF oncogene are under investigation (24).

1.2.4. High-Grade Gliomas

High-Grade Gliomas (HGG) comprise glioblastoma (WHO grade IV), anaplastic astrocytoma (WHO grade III), mixed anaplastic oligoastrocytoma (WHO grade III) and anaplastic oligodendroglioma (WHO grade III) (34).

Although anaplastic astrocytoma can be diagnosed as a *de novo* tumor, in the majority of patients (50-75%) it represent 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 a 5-10% 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 LGG. To confirm the role of TP53 in the glioma's evolution, it's emphasized that 90% of patients with this protein mutation in the tumor relapse, already presented TP53 mutation in the primitive LGG.

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 the 25% if the cases), co-deletion chromosome 1p19q (in 15% of the cases), mutation in IDH1/2, as well as the tumor-suppressor gene PTEN (15%), and finally the amplification of EGFR in 10% of the patients.
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 22 (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 LGG, indicates that anaplastic astrocytomas are the transitional form between LGG and secondary glioblastoma multiforme. Nevertheless, the pathogenesis of primary anaplastic astrocytomas is still unknown.
Glioblastoma multiforme is the most common and aggressive form of malignant astrocytoma and can arise *de novo* or from pre-existing lower grade tumors (18). The incidence of glioblastoma in Italy is of 2-3 new cases every 100.000 people per year (23). Glioblastoma can occur at any age but is more likely to develop in older people (median age 53 years). It is predominantly found in male. Although in adults glioblastoma 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 (35). It is generally associated with a poor prognosis (mean survival 11 months), yet individual patient survivals may vary.
Historically, glioblastoma have been classified into two groups: “primary” and “secondary” glioblastoma (36). The vast majority of glioblastoma (approximately 90%) develop rapidly *de novo* in elder patients, without clinical or histological evidence of a less malignant precursor lesion (primary glioblastoma) (37). Secondary glioblastoma progress from low-grade diffuse astrocytomas or anaplastic astrocytomas. 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 profile (Figure 1.2.1). Decisive genetic signpost of secondary glioblastomas are IDH1 mutations (38), that are absent in primary glioblastomas and which are associated with a hypermethylation phenotype (37). IDH1 mutations are the earliest detectable genetic alterations 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 (37). According to Yan and collaborators, the mutation of IDH1 promote the progression of low-grade gliomas into high-grade (39).

Clinical differences have been reported between the two groups, with secondary glioblastomas occurring predominantly in younger patients (45 years versus 60 years for primary glioblastomas) (40, 41).

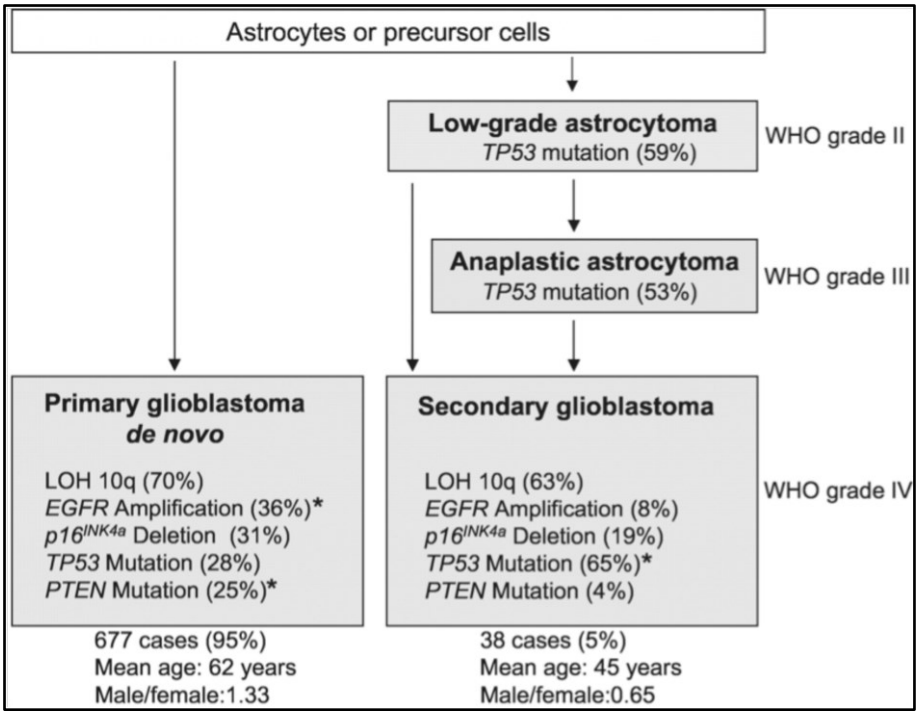

Figure 1.2.1. Genetic alterations in primary and secondary glioblastomas. From: (39).

Recently, advances in molecular technologies, especially high-density microarray and genome sequencing, have made it possible to evaluate genetic and epigenetic changes in glioblastoma at the genome-wide level (42). Specifically, these integrative genomic studies provided a comprehensive view of the complex genomic landscape of glioblastoma, revealing a set of core signaling pathways commonly activated in glioblastoma (Figure 1.2.2): the p53 pathway, the RB pathway and the RTK pathway (42). The majority of glioblastoma tumors have genetic alterations in all the 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 (42). Moreover, these studies have revealed the role of novel pathways, such as the involvement of NF1 mutations in 23% of sporadic glioblastoma, the heterozygous deletion of the NF-kB inhibitor a (NFKBIA) gene in 25% of glioblastoma samples, and the previously mentioned mutation of IDH1 in 12% (42).

Importantly, starting from these data, some Authors tried to molecularly classify glioblastomas into clinically relent subgroups. For example, Verhaak et al. performed unsupervised clustering analysis of the available glioblastoma data set and grouped the tumors into four subtypes proneural, neural, mesenchymal and classic (43). Interestingly, these subtypes were characterized by specific genetic

alterations. Moreover, the prognostic value of the molecular sub-classification has also been evaluated with several studies suggesting that gliomas with the expression of genes associated with neurogenesis (proneural subtype) generally correlate with marginally improved survival, while gliomas with mesenchymal gene expression have a poorer outcome (1 year for proneural versus 6 months for mesenchymal) (44). Moreover, although gliomas with proliferative o mesenchymal characteristics generally have a worse outcome, several studies have confirmed that these subtypes are also more sensitive to combinational radiation and chemotherapy (43).

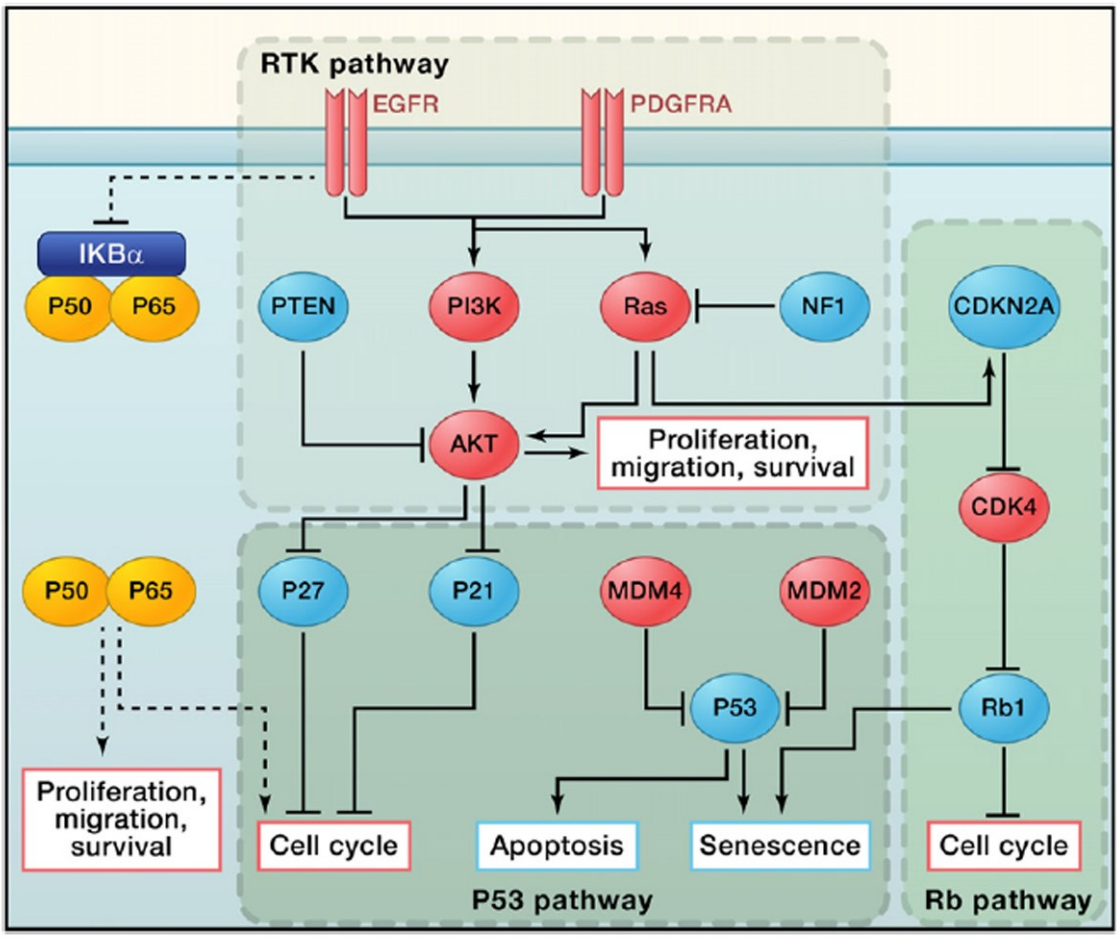

Figure 1.2.2. Core signaling pathways in glioma tumorigenesis. From: (42).

Therefore, in HGG, genetic studies have contributed to dissect the basis of glioblastoma heterogeneity and to demonstrate that, despite a similar histological appearance, different classes of diseases can be identified on molecular basis. This novel approach would allow a more precise classification system that accurately reflect the cellular, genetic and molecular basis of gliomagenesis, and can be a prerequisite for identifying subsets of patients characterized by different prognosis and by a distinct responsiveness to specific adjuvant therapies. In conclusion, this would ultimately allow achieving individualized clinical care of glioma patients (44).

1.3 THE HETEROGENEITY OF GLIOMA CELLS

Phenotypic and functional heterogeneity arise among cancer cells within the same tumor as a consequence of genetic alterations, environmental differences and reversible changes in cell properties. Some cancer cells also contain a hierarchy in which tumorigenic cancer stem cells differentiate into non-tumorigenic progeny.

High-grade brain tumors are heterogeneous with respect to the composition of *bona fide* tumor cells and with respect to a range of intermingling parenchymal cells. Glioblastoma harbor multiple cell types, some with increasing tumorigenicity and stem-like properties (45).

1.3.1. Cancer Stem Cells: from the "Stochastic Model" to the "Hierarchical Model"

Clinical and experimental observations demonstrated a frequent failure in inducing a tumor in animal model injected with the bulk tumor cells (45). The Cancer Stem Cell (CSC) hypothesis postulates that a small subpopulation of cancer cells possessing self-renewal characteristics is responsible for initiating and maintaining cancer growth. According to the CSC model, the large populations found in a tumor might represent diverse stages of differentiation (Figure 1.3.1). The biological characteristics shared by normal stem cells and CSCs mainly involve self-renewal and differentiation potential, survival ability, niche-specific microenvironment requirements, and specific homing to injury site and may have important implications in terms of new approaches to cancer, since CSCs represent a novel and unexplored therapeutic target (46, 47).

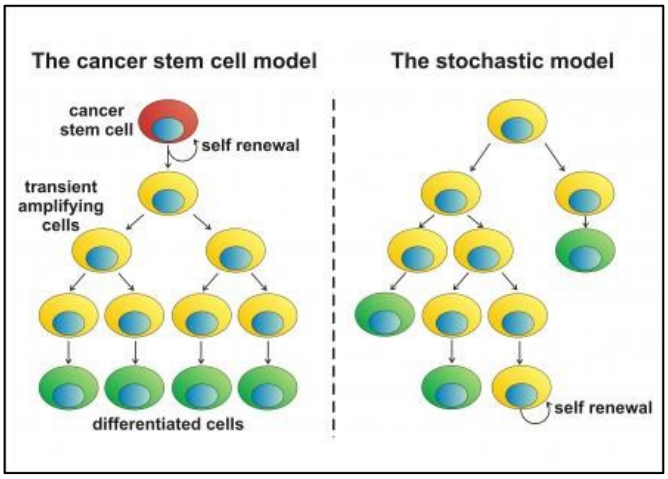

Figure 1.3.1. Cancer Theories. The Cancer Stem Cell Theory (i.e. Hierarchical Model, or Deterministic Model) suggests a clear hierarchy of cells within a tumor. The Stochastic Model affirms that tumor growth is a random process to which all cells can contribute. From: "Cancer: a disease of stem cells?" 2011 in http://www.eurostemcell.org/de/node/16637.

Bonnet and Dick gave the first demonstration of the existence of CSCs in 1997 in human acute myeloid leukemia (48). Authors demonstrated that a subpopulation of leukemia cells that were $CD34^{+}/CD38^{-}$, were able to self-renewal and to generate, when injected in NOD-SCID mice (non-obese and diabetic - severe and combined immunodeficient systems), a myeloid acute leukemia phenotypically identical to the starting tumor. Authors named these cell "tumor-initiating cells" (TICs) and demonstrated that the phenotype of these cells was very similar to the ones described for normal hematopoietic stem cells, suggesting that tumor and normal tissue are governed by a similar hierarchical model.

Therefore, cells inside the tumor bulk are not all the same in terms of self-renewal, proliferating and differentiation ability, and only the very primitive cells are characterized by the attitude to initiate and maintain the tumor and can be responsible of recurrences and metastasis (49).

This leukemia model represented the paradigm for the subsequent studies focused on solid tumors, such as mammary carcinoma (50), glioma (51, 52), melanoma (53), thyroid cancer (54), lung cancer and gastroenteric tumors (55).

Solid tumors resulted to be more complex as they contain not only tumor cells, but also stromal cells, which interactions can change the first one's properties.

Notably, CSC share several stem cell related features with non-neoplastic stem cells, especially the ones related to self-renewal (56).

In fact, pathways related to Wnt, Sonic Hedgehog and Notch, which normally regulate the cell self-renewal, are present and up-regulated in several cancers (57).

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 (45, 58).

Regarding the origin, there are three hypothesis on the origin of cancer stem cells (Figure 1.3.2) (45):

- CSC may result from a normal stem cell that undergoes several successive mutations and acquire a neoplastic phenotype;
- CSC may originate from a precursor cell that, by mutation, re-acquire self-renewal properties.
- CSC may originate that, by mutation at a level of specific cell signaling pathway, may return to proliferate and re-acquire self-renewal potential.

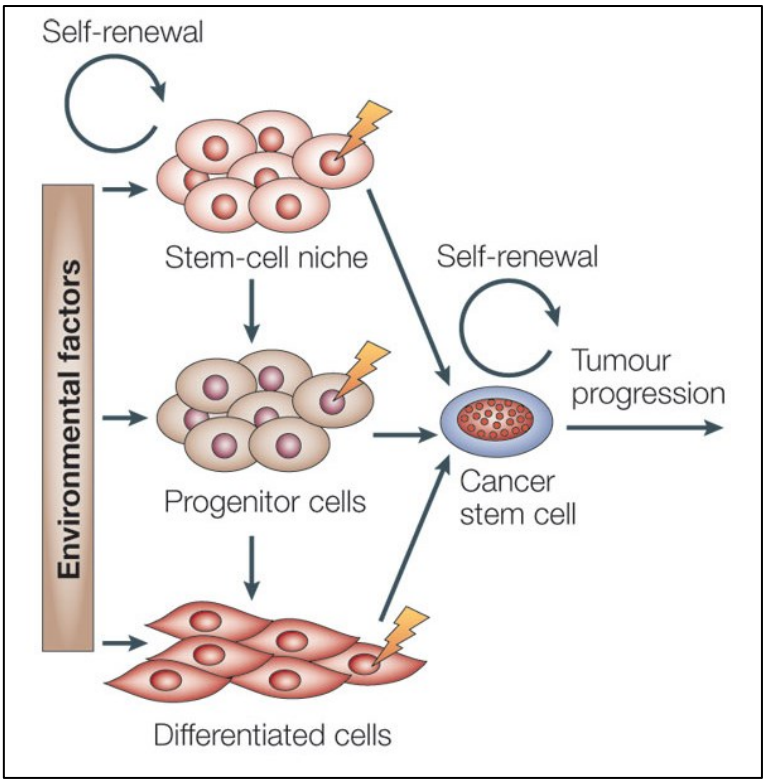

Figure 1.3.2. The origin of Cancer Stem Cells. The cancer stem cell may appear after mutation in specific stem cells or early stem cell progenitors. It is also possible that cancer stem cells can be derived from differentiated cells. There might be numerous factors in the host microenvironment that trigger the initial steps of tumor formation. From: Bjerkvig et al. The origin of the cancer stem cell: controversies and new insights. 2005 Nature Reviews Cancer 5, 899-904; doi:10.1038/nrc1740.

Recently, the stem cell theory for cancer has been questioned. Specifically, Quintana showed that modified xenotransplantation assay conditions, including the use of more 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 cancer, tumorigenic cells are quite frequent (59).

One of the possible explanation for this results is the fact that the tumor microenvironment (stromal fibroblasts, adipocytes and endothelial cells, as well as the extracellular matrix) and the immune system play an important role in cancer progression (60, 61). Consequently, the xenograft model could not offer an appropriate microenvironment for the growth of human tumors, because of the differences between mice and humans and the lack of an intact immune system. These limitations can be important when evaluating the tumor-initiating capacity of human cancer cells. Thus, it is possible that the sub-population of cells that appeared non tumorigenic in a NOD-SCID mouse model might actually be tumorigenic in the presence of the appropriate microenvironment. In other words, tumor cells may be functionally homogeneous, with heterogeneous potential arising as a consequence of extrinsic cues or of their lack.

Another important aspect is to understand how cancer stem cells are able to resist to common therapies and treatments, and it would be clinically useful to assess the survival based on the number of CSCs and not on the residual tumor mass (58).

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 remain majority of 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 cells responsible for tumor self-renewal.

1.3.2. Glioma-Initiating Stem Cells

The bulk of malignant cells in glioblastoma is generated by rare fractions of self-renewing, multipotent tumor-initiating cells (51, 52), responsible for tumor growth and recurrence and resistance to chemo- and radiotherapies (62). Glioma Stem Cells (GSCs) generate tumors with the cardinal features of the glioblastoma from which they are derived, including an infiltrative phenotype and histopathological feature such as hypercellularity, pseudopalisading necrosis, and angiogenesis.

The first report of cells with stem-like properties in brain tumors was provided by Ignatova at al., where surgical specimens of glioblastoma multiforme were shown to have clonogenic neurospheres-forming cells that expressed both neuronal and glial markers upon differentiation (63). According to Vescovi's definition (64), brain tumor cells could be considered as stem cells if they show:

- Cancer-initiating ability upon orthotopic implantation;
- Extensive self-renewal capacity either *ex vivo* or *in vivo*;
- Karyotypic or genetic alterations;
- Aberrant differentiation properties;
- Capacity to generate non-differentiated end cells;
- Multi-lineage differentiation capacity.

Subsequently, Dirks et al. demonstrated CSC in brain tumors by transplantation of $CD133^+$ or $CD133^-$ cell populations into immunodeficient mouse. With as few as 100 $CD133^+$ cells from the primary tumor, a new phenocopy of the tumor could be created in the transplanted mice, whereas unsorted or $CD133^-$ primary tumor cells were unable to cause *de novo* tumor generation. As part of

what has come to define CSC self-renewal capacity, was also shown the ability of serially transplanted $CD133^+$ cells to recapitulate the original tumor (Figure 1.3.3) (52).

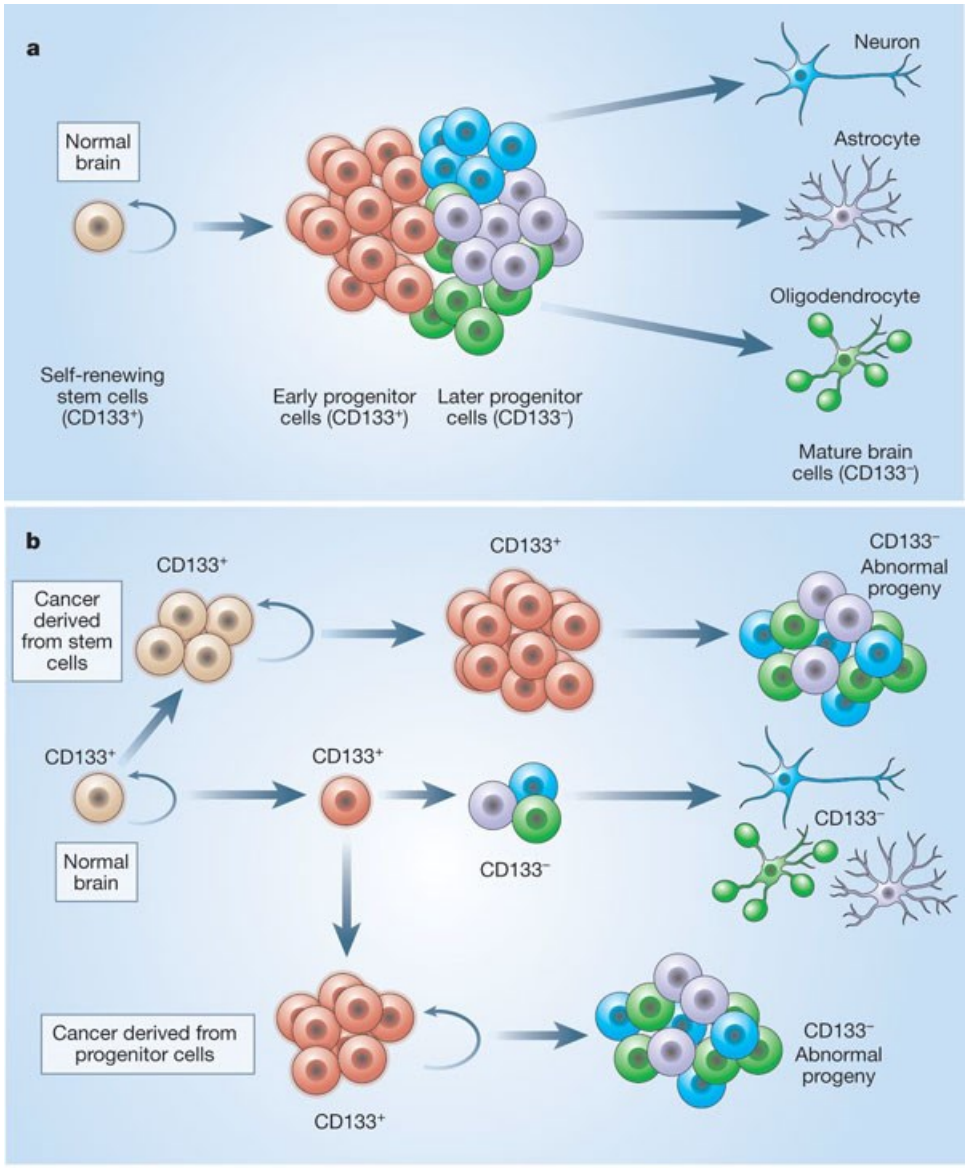

Figure 1.3.3. CD133 as a marker of brain stem cell (A) and glioma-tumor initiating cell (B). From Clarke MF. Nature 2004:432, 281-282

Nowadays, several methods have been applied to isolate GSC. Singh et al. proposed a prospective isolation of a sub-population of cells expressing the cell surface marker CD133 (52). This latter, formerly known as PROM-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 (65). Although no interacting proteins are known, a role in cell polarity and cell migration was suggested due to his specific localization. When isolated from human brain tumors, $CD133^+$ cells display stem-like properties *in vitro*, such as enhanced

proliferation ability, self-renewal, differentiation and neurosphere-like growth (52). Indeed, $CD133^+$ cells are preferentially resistant to chemotherapeutic agents and radiation and express high levels of ABC-transporter BCRP1, (O)6-methylguanine DNA methyltransferase and negative regulators of apoptosis (62, 66). However, recent reports indicate that this initially proposed model might represent an over-simplification and stem-cell specificity of the epitope detected by the antibody AC133 has been questioned (67, 68). Thus, stem cell-specific markers other than CD133, as CD15/SSEA-1 and integrin α-6, have been described, but there is not yet consensus for optimal markers for GSC in glioblastoma (69-71).

Another 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 (72). 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 (52) or characterized by generation of cells co-expressing glial and neuronal markers (63). Importantly, neurospheres expressed many genes characteristic of NSC-derived spheres, such as CD133 (73).

Subsequently, Galli et al demonstrated that glioblastoma-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. Importantly, tumor xenograft could be serially transplanted thus confirming the in vivo self-renewal and tumorigenic capacity of neurospheres (85). Many papers have subsequently confirmed the results obtained by Galli (83) and the superiority of the serum-free culture-method above the standard serum-supplemented culture conditions (102).

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 (74).

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 (67). Moreover, neurospheres can be effectively obtained only from 30-50% of glioblastoma, but not from adult low-grade gliomas (51, 73). Unfortunately, these methods are mostly effective in glioblastoma and

isolated/obtained cells cannot always be analyzed by high-throughput methods thus making more difficult their study.

In order to overcome limitations of the neurosphere-based culture of GSC, Pollard et al. recently reported a methodology for deriving and expanding glioma neural stem cell lines in adhesion onto a laminin coating (75). Adherent cultured GSC are characterized by better features compared to neurosphere cultured cells: they can be isolated from a higher number of tumor samples, can be expanded with higher efficiency in order to obtain a higher yield, possess a lesser propensity to the spontaneously differentiation due to accessibility of growth factors by inner cell mass of neurosphere and maintain the ability to be tumorigenic in orthotopic transplantation and are suitable for genetic and drug screenings.

1.3.3. Expression of radial glia markers in gliomas

Numerous studies suggest that astrocytomas may arise from pre-astrocytic transitional cells or multipotent neural stem cells (76), and the observation that glioblastoma tissues are characterized by the expression of molecular markers distinguishing the early stages of development, has attracted much interest. An example is provided by the expression in gliomas of radial glial cells markers, such as fatty acid-binding protein 7 (FABP-7).

Radial glial cells (RGC) play at least two crucial roles in the cortical development: neuronal production in the ventricular zone, and the subsequent guidance of the neuronal migration toward the cerebral cortex (77). FABP-7, also known as brain lipid binding protein (BLBP), is a member of the FABP family, consisting of structurally related proteins that have specific cell, tissue, and development patterns of expression. In the brain tissue, FABP7 gene is expressed more abundantly at an immature stage of the brain than after maturation (78). Potential FABP-7 binding partners are decosahexaenoic (DHA), oleic, linoleic and elaidic acid (79). Generally, FABP proteins are involved in the uptake and intracellular trafficking of fatty acids, bile acids and retinoids, as well as in cell signaling, gene transcription, cell growth, and differentiation. In radial glial cells (RGC), FABP-7 plays a role in the establishment of the radial glia system required for neuronal migration (80).

As reported by Liang et al, the nuclear expression of the radial glial marker FABP-7 is associated with poor survival in patients with glioblastoma (81). Although FABP-7 is a cytoplasmic protein, its varying subcellular location between nucleus and cytoplasm has been reported in developing brain (82), glioma cells (83) and glioblastoma specimens (84). In addition, Taylor et al found that

all ependymoma-derived tumor spheres displayed a CD133+/Nestin+/FABP-7+ immunophenotype similar to that of RGC, suggesting that these cells may be the cells of origin of ependymomas (85). However, since the increased FABP-7 expression was also found in glia following nerve injury (84), it is not yet clear whether the re-expression of radial glial markers occurs only in glioma cells, or may be even in the stromal cells.

1.4 THE TUMOR MICROENVIRONMENT

In addition to malignant cancer cells, tumors contain a variety of different stromal cells that constitute the so-called *tumor microenvironment*. Cancer cells and resident or infiltrating stromal cells are involved in heterotypic interactions with one another. Some of these cell types provide crucial support for tumor growth, are responsible for inducing angiogenesis, avoiding immune response against tumor cells, deregulating cellular energetics or inducing invasion and metastasis (86). Thus, this *tumor-associated stroma* is not a passive spectator of tumor growth, but it plays an active role in tumorigenesis and cancer progression (87).

1.4.1. Tumor-Associated Fibroblasts

Nowadays, the tumor microenvironment has been studied mainly in solid tumors of epithelial origin and Authors focused their attention on Cancer Associated Fibroblasts (CAF) or Tumor Associated Fibroblasts (TAF) that represent the most abundant cell type in the tumor stroma (88-90).
Five aspects can define TAF (Figure 1.4.1):

- 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 Mesenchymal Stem Cells (MSC);
- Expression of proteins involved in processes of invasion and remodeling of surrounding stroma such Matrix metalloproteinases (MPPs), stromelysin-1 (SL-1), thrombospondin-1 (TSP-1) and Tenascin-C (TN-C);
- Expression of proteins associated with neovascularization such as Smooth Muscle Actin (SMA), desmin, Stromal-cell Derived Factor-1 (SDF-1) and Vascular Endothelial Growth Factor (VEGF);
- Production of growth factors such as 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;
- Secretion of chemokines, such as Monocyte Chemotactic Protein 1 (MCP1) (91).

Although the biological origin of TAF is still undetermined, data in the literature currently support four possible origins (92):

- The recruitment of resident tissue stem cells;
- Epithelial to mesenchymal transition (EMT) of the tumor parenchyma;
- Fibroblast recruitment into the tumor stroma;
- Recruitment of bone marrow-derived cells from the circulation (93).

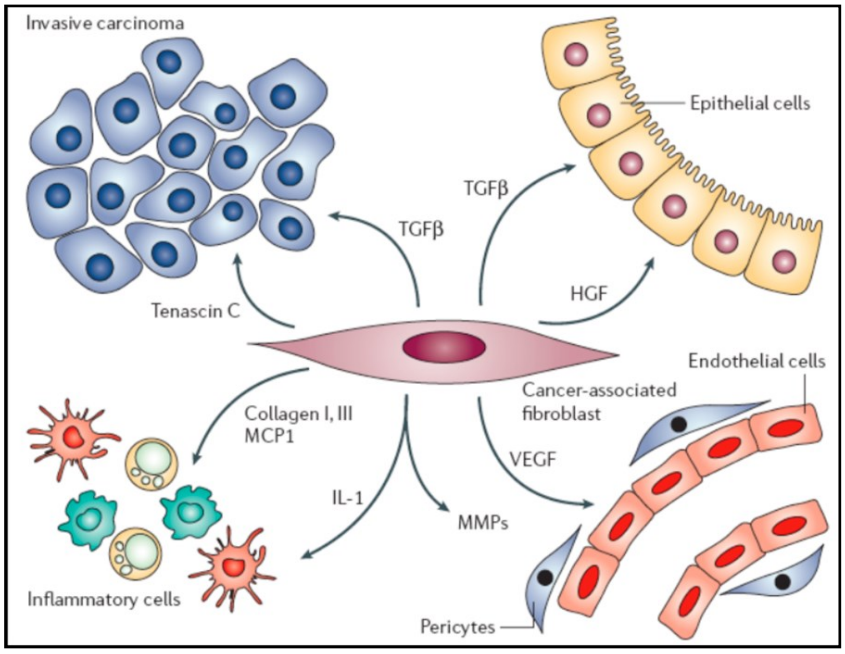

Figure 1.4.1. Factors produced by TAF and their targets within the tumor microenvironment. From: (94).

Particularly interesting are the evidences pointing to a possible role played by either bone marrow-derived or resident mesenchymal stem cells (95), since these latter can originate many of the cell types present in the tumor microenvironment, can migrate to sites of damage and are able to induce immune tolerance.

The mechanisms that lead to establish the TAF phenotype are not clear yet. Fibroblasts are usually activated during injury, but, upon healing, they lose their activated state. In contrast to wound healing, TAF 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" (96, 97). This activation can be induced by various factors released during injury, including growth factors such as Transforming Growth Factor-β (TGF-β), Platelet-Derived Growth Factor (PDGF), EGF and bFGF, which are released from injured epithelial cells and infiltrating mononuclear cells such as monocytes and macrophages (94).

In addition, fibroblasts are activated by direct cell-cell communication and contacts with leukocytes by adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) or vascular-cell adhesion molecule-1 (VCAM-1) (94). Fibroblast activation can also be achieved by through

reactive oxygen species, complement factor C1 and or altered extracellular matrix (ECM) composition (94). Moreover, during tumorigenesis, following loss of basement membrane integrity, tumor cells invade into the underlying connective tissue and directly interact with local mesenchymal fibroblasts.

Importantly, the mechanisms that determine a permanent activation of fibroblasts in tumors are still unknown, although epigenetic mechanisms have been hypothesized (88, 90). The essential role of TAF in the tumor progression suggested the possibility to envision TAF as possible novel therapeutic target. The advantages of using the stroma as a therapeutic target comprises that these cells are no genetically unstable as cancer cells and they are less likely to develop resistance to treatment and to therapy (98).

An interesting approach is to inhibit TAF by blocking molecules responsible for their activation. For example, inhibition of TGF-β by specific molecules seems to reduce the incidence of metastases (99). Another possible target is FAP, thought to be involved in the control of fibroblast growth or epithelial-mesenchymal interactions during development, tissue repair and epithelial carcinogenesis (94). Further studies are suggested the use of Bevacizumab, a recombinant humanized anti-VEGF monoclonal antibody, able to inhibit tumor angiogenesis (100).

In any case, it is very important to notice that these 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 brain tumor microenvironment

The composition of the tumor microenvironment varies depending on the tumor site. The brain, in particular, consists of numerous specialized cell types such as microglia, astrocytes and brain endothelial cells. In addition to these brain-resident cells, brain tumor have also been shown to be infiltrated by different populations of bone marrow-derived circulating cells (Figure 1.4.2) (101). However, the role of different cell types that constitutes the tumor microenvironment in the progression of gliomas is only poorly understood.

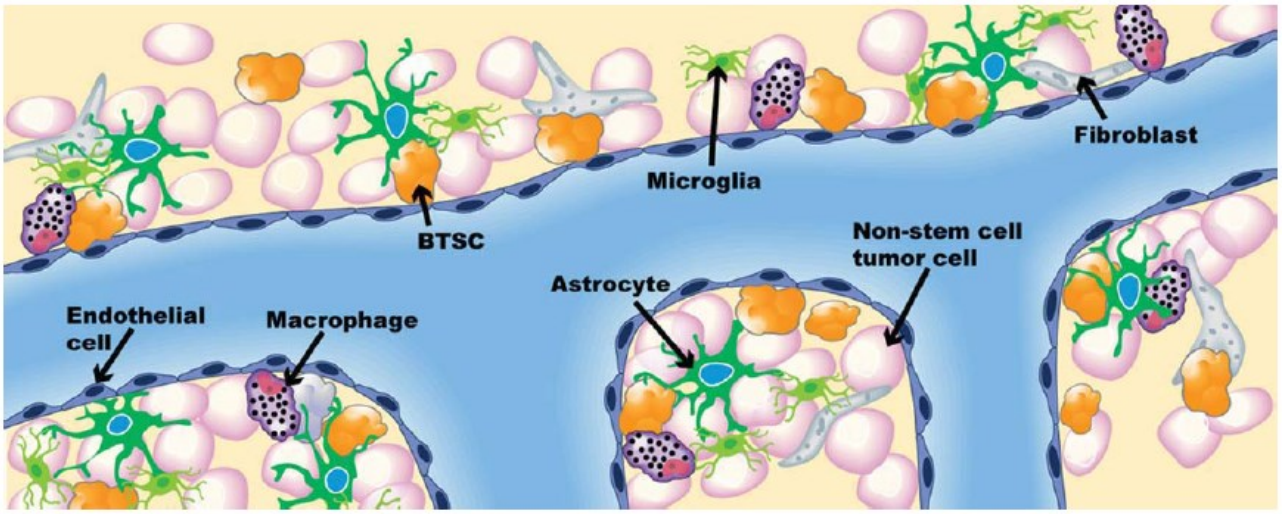

Figure 1.4.2. The glioblastoma microenvironment. The microenvironment of glioblastoma is composed of several stromal cell types, which are believed to make distinct contribution to tumor progression and invasion. From: (102).

Microglia-macrophages

The majority of non-transformed cells in the glioma tissue is represented by tumor-associated macrophages (103, 104). Resident macrophages of the brain are termed microglia. These cells invade the brain early in development and differentiate into so-called, resident ramified microglia (105). The precise origin of tumor microglia remain to determined, since it is not yet clear whether these cells are recruited *de novo* from the microglial population resident in the brain or, alternatively, migrate into brain tumors from the periphery (106).

Candidate chemoattractants of tumor-associated microglia include monocyte chemotactic protein-3 (MCP-3), colony stimulating factor-1 (CSF-1) and hepatocyte growth factor/scatter factor; each of these has been shown to be released by gliomas and microglia are known to express receptors for these chemoattractant factors (107-109)

Immune functions of microglia can be suppressed when these cells are located in the glioma environment similarly as described for other tumors (103). Glioma cells produce cytokines with an anti-inflammatory function such as IL-10, IL-4, IL-6, TGF-β and prostaglandin E (110). Moreover, glioma-associated microglia also appear to express low levels of the MHC class II molecule (111).

The first identification that microglia might modulate the biology of glioma cells came from cell culture studies, in which microglia promoted the migration of GL261 mouse glioma cells (112). The impact of microglia on glioma migration might relate to the production of membrane type-1 metalloproteinase (MT1-MMP or MMP-14) that are produced by microglia in response to soluble factors secreted by glioma cells. Glioma cells also release metalloprotease 2 (MMP-2) that is fully activated by MMP-14. The consequent degradation of the extracellular matrix has been postulated to enhance the invasion of glioma cells into the brain parenchyma (113).

Endothelial cell, pericytes and the perivascular niche

The tumor vasculature contributes an additional large compliment of non-malignant cells to gliomas. Gliomas are highly vascularized tumors and emerging evidences indicate that endothelial cells, pericytes and astrocytes that form the neurovascular unit function to support tumor progression (102). This microanatomical structure has received increasing attention in the last few years following the discovery that the perivascular niche (PVN) is the stem cell niche in both normal and malignant brain tissue.

The vasculature in gliomas is characterized by hyperplasia and microvascular proliferation (MVP) that are histological hallmarks of high-grade gliomas (114). The presence of MVP is associated with tumor transition to a malignant phenotype. Microvascular proliferating structures have been shown to derive from hyper-proliferative activity in a combination of endothelial, pericytic and vascular smooth muscle cells (115). MVP structures represent regions of angiogenesis, which is critical for tumor progression and is particularly important for brain tumors, especially malignant gliomas that are among the most vascularized/angiogenic tumors. These angiogenic regions, of which the perivascular niche is a part, have emerged as important locations responsible for the maintenance of glioma stem cell (GSC) populations (116).

GSC are intimately associated with the vasculature across multiple brain tumor types. Endothelial-derived factors also maintain self-renewal properties of glioblastoma stem cells *in vitro*. When cultured with primary endothelial cells, GSC preferentially associated with them(117).

The development of the brain tumor vascular niche environment requires the recruitment of bone marrow-derived cells, and some of these include: 1. Endothelial progenitors that mature into endothelia of the vascular lumen; 2. Pericyte progenitor cells that envelope the vasculature and mature into pericytes and vascular smooth muscle cells; and 3. $CD45^+$ vascular-associated cells, some of which give rise to perivascular macrophages and microglia (118).

In the context of tumor development, recruitment of pericytes and vascular smooth muscle cells to the tumor is critical for structural stability of the vascular niche and for survival of tumor-endothelial cells (119).

Fibroblasts and mesenchymal cells

Activated stromal fibroblasts have been identified by several reports to play a key role in the induction of angiogenesis and metastasis in melanoma and other solid tumors (120, 121). A similar role within the glioma microenvironment has also been suggested. Co-culture of brain-derived

human fibroblasts with glioblastoma cells induced production and activation of MMP2, and its activators MT1-MMP and MT2-MMP, that are involved in the progression of gliomas (122).
Recently, our group identified a novel population of mesenchymal stem cells which can be isolated from human low-grade and high-grade gliomas. This cells, called Glioma-Associated Stem Cells (GASC), although devoid genetic alterations of the tumor of origin, are characterized by stem cell properties, aberrant growth features, a gene expression profile typical of tumor-supporting cells and are able to enhance *in vitro* the aggressiveness of glioblastoma initiating stem cells by means of exosome release (123).

Astrocytes

Astrocytes are often identified by their expression of Glial Fibrillary Acidic Protein (GFAP), which increase in response to injury. Astrocytes were originally thought to play a passive role in the brain, but are now known to play critical functions in supporting the integrity of the blood brain barrier (BBB) (124), in the transmission of neural impulses and in maintaining the biochemical homeostasis of the brain tissue (125). In addition, astrocytes contribute to the brain's response to injuries. When injury occurs, astrocytes become reactive, change their morphology, proliferate and migrate to the area of injury (126).
In addition to reactive astrocytes, adult Neural Stem Cells (NSC) also express GFAP (127). These cells share ultrastructural and protein expression characteristics with mature astrocytes (128) and reside in two neurogenic niches within the adult brain: the sub-ventricular zone of the lateral ventricles and the sub-granular zone in the dentate gyrus of the hippocampus (129). Within these regions, neural stem cells reside adjacent to blood vessels, and these adult astrocyte-like stem cells proliferate slowly and are thought to divide asymmetrically to give rise to progenitor cells that proliferate rapidly and symmetrically to produce terminal cell types such as neurons and glia (130).
Recent studies have highlighted the plasticity of astrocytes with regard to their functions in injury and as stem cells. In addition to shared markers and ultrastructural characteristics between reactive astrocytes and adult NSC, recent works have demonstrated that reactive astrocytes derived from an injury setting possess characteristics specific to an adult NSC, including the expression of NSC markers as well as the ability to proliferate and generate neurospheres (124, 131-133). This studies suggest a novel role for astrocytes in the context of injury and demonstrate the plasticity of reactive astrocytes.
The role of astrocytes cam vary significantly among gliomas. Astrocytes are closely associated with the vascular endothelium and they secrete a number of neurotrophic factors, including transforming growth factor-α (TGF-α), CXCL-12, SIP and GDNF, that have been described as capable of driving

the invasive properties of glioblastoma cells (134). Reports suggest that glioma-induced remodeling within the tumor microenvironment through disruption of the BBB facilitates tumor invasion and involve abnormal astrocyte-endothelial interaction (135).

Tumor-astrocyte interactions are an important mechanism by which glioma cells facilitate tumor invasion. An increase in MMP2 production following cross-communication between co-cultured astrocytes and glioblastoma cells, gas been associated with an enhancement of the invasive capacity of glioblastoma cells through production of proMMP. This effect was mediated by the urokinase-type plasminogen activator (uPA)-plasmin cascade. Astrocytes produced significant amount of proMMP2, the inactive form of the matrix metalloproteinase MMP2, as well as uPA and its receptor uPAR, both required for plasminogen cleavage. Plasminogen provided from glioblastoma cells were cleaved to produce plasmin, which cleaved proMMP2 to its active form MMP2. Furthermore, analysis of resected human glioblastoma specimens revealed elevated levels of plasminogen, suggesting that a similar mechanism may exist *in vivo* (136).

Astrocytes also mediate activation of developmental pathways like the Sonic Hedgehog (SHH) pathway, which is known to play an important role in GSC self-renewal and growth of gliomas. SHH signaling was shown to be activated in gliomas and was correlated with increasing grade (137).

1.5 EXOSOMES

Many kinds of cells shed small vesicles that play a key role in the intercellular communication. Three major kind of vesicles have been described so far: microvesicles (100 nm to 1 μm in diameter), which directly bud from the plasma membrane, apoptotic blebs (50-500 nm), which are released by cells undergoing apoptosis, and exosomes (30-150 nm), released via exocytosis from multivesicular bodies (MVBs) of the late endosome (138, 139).

Following the initial description in reticulocytes (140), several cell types have described to release exosomes in the extracellular medium *in vitro*, such as hematopoietic cells (B-, T-, dendritic and mast cells and platelets), epithelial cells, neural cells (oligodendrocytes, neurons, microglia and Schwann cells), adipocytes, fibroblasts, stem cells and many types of tumor cells. Of relevance, exosome are found *in vivo* in several biological fluids, such as saliva, urine, plasma, seminal fluid, amniotic fluid, ascites, bronchoalveolar lavage fluid, synovial fluid, breast milk and cerebrospinal fluid (CSF) (141).

1.5.1. Biogenesis and secretion

Exosomes originate in the intraluminal vesicles (ILVs) of MVBs, which fuse with the cell membrane in an exocytic manner (142): as late endosomes bud off parts of their limiting membrane into the lumen of late endosomes, vesicles are formed inside the lumen (ILVs) originating this particular type of late endosome, which accumulates hundreds of ILVs and is termed MVB (Figure1.5.1).

Initially, it was thought that MVBs could just fuse with lysosomes to degrade their intraluminal content. This process was considered to be important to remove transmembrane proteins as well as for the recycling system of cellular membranes (143, 144). Later it was demonstrated that in reticulocytes undergoing maturation into red blood cells, MVBs can also fuse with the plasma membrane to release their ILVs into the extracellular space, and the term “exosomes” was proposed to identify these extracellular intra-endosomal vesicles (140).

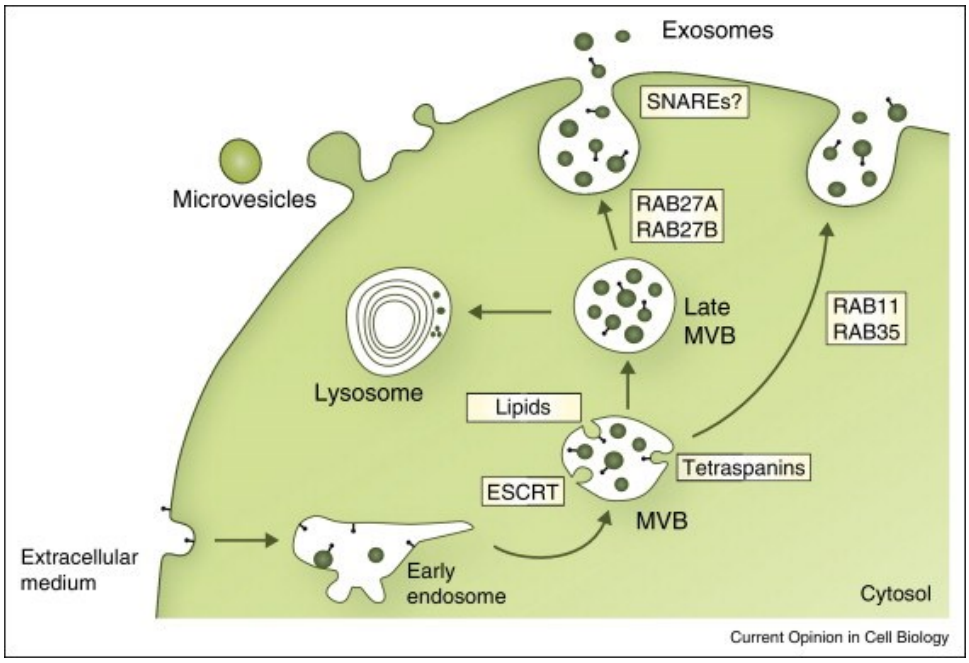

Figure 1.5.1. Biogenesis and secretion of exosomes. From (145).

The process of MVBs formation is coordinated by the Endosomal Sorting Complex Required for Transport (ESCRT). This complex consists of four soluble multiprotein complexes, called ESCRT-0, -I, -II and –III, and it is usually recruited to the cytosolic side of the endosomal membrane for the sorting of selected proteins to ILVs. This process is known to require ubiquitination of proteins, which are loaded into ILVs (146). While the ESCRT proteins are clearly required for the targeting of membrane proteins for lysosomal degradation, the function of the ESCRT machinery in the formation of ILVs secreted as exosomes is less clear. As a matter of fact, the identification by proteomic analysis of members of the ESCRT complex , such as Alix and Tumor Susceptibility Gene 101 (Tsg101), in dendritic cell derived exosomes strongly support an ESCRT-dependent exosome biogenesis (147).

Most recently, an ESCRT-independent mechanism was described involving the sphingolipid ceramide. Ceramide is generated during cellular stress and apoptosis either by *de novo* synthesis or by sphingomyelinase, the enzyme that hydrolyzes sphingomyelin into ceramide. Ceramide contributes to the cellular signaling by playing a role in membrane microdomain coalescence, receptor clustering, vesicle formation, membrane fusion/fission and vesicular trafficking (148). Additionally, ceramide is enriched in exosome membranes. Further validating the ESCRT-independent mechanism, inhibition of the neutral sphingomyelinase decreased exosome formation and release, whereas depletion of different ESCRT components did not reduce exosome secretion or the formation of MVBs (149).

Interestingly, exosomes produced by the ESCRT-independent pathway are enriched in tetraspanins, which are transmembrane proteins that may also be involved in endosomal sorting pathways (150). Based on these observations, ESCRT-dependent sorting mechanisms may target proteins loaded into ILVs for lysosomal degradation, whereas ESCRT-independent sorting mechanisms may target ILVs for secretion. However, this process is much more complex and may depend on cell type, nature of the cargo or other stimuli.

Exosomes are secreted by the fusion of MVBs with the plasma membrane and released into the extracellular space. Exosome secretion is not considered a random event, but rather a highly controlled process. Although largely is still under investigation, this process is thought to be coordinated through the transport and fusion of MVBs with the plasma membrane by the microtubule and actin cytoskeleton, t- and v-SNAREs and Rab GTPases (151). Rab GTPases are ubiquitously expressed proteins that are responsible for the coordination of vesicle trafficking events (152). For example, over-expression of Rab11 has been shown to stimulate exocytosis and Rab27a and Rab27b control different steps of exosome secretion pathway (153, 154).

Exosome secretion may be enhanced by many different conditions. For example, has been demonstrated that cancer cells cultured in hypoxic or low pH conditions showed an enhanced release of exosomes (155).

In order for secreted exosomes to exert any biological function, they must be adsorbed by and deliver their contents to a recipient target cell. However, the specific targeting of exosomes to target cell and how this process unfolds in normal physiology or in the diseased state is not well understood. This process must critically depend on the specific adhesion molecules, integrins and antigenic factors expressed on the exosome, as well as the receptor or other docking molecules found on the surface of target cells. For example, exosomes from T-, B- and dendritic cells were shown to communicate with antigen presenting cells by transferring their content in a n unidirectional manner and modulating gene expression in the recipient cell (156).

Presumably, any cell capable of endocytosis or phagocytosis may participate in the uptake of exosomes. Indeed, many studies have documented that exosomes can be internalized by recipient cells through many processes, such as receptor-mediated endocytosis (both clathrin- or dynamin-dependent), phagocytosis and pinocytosis (157, 158).

However, the mechanisms of uptake of exosomes by recipient cells have not yet been completely clarified.

1.5.2. Exosome components

Apart from their morphological and physical properties, exosomes are usually identified on the bases of their unique protein and lipid composition. Because of their endosomal origin, exosomes contain proteins involved in membrane transport and fusion (e.g. Rab GTPases, annexins, flotillins), MVB biogenesis (e.g. Alix, Tsg101) or protein families mostly associated with lipid microdomains, such as integrins and tetraspanins (e.g. CD9, CD63, CD81, CD82) (142).

According to proteomic studies, exosome protein composition is different in membrane vesicles released by different cell types (147). Specifically, exosome protein content includes both conserved proteins, i.e. identified in almost all exosomes despite their origin, and cell-type specific proteins. Among conserved proteins, the heat shock protein cognate 70 kDa protein (hsp70) and the tetraspanin CD63, are the most frequently identified (141). Moreover, other frequently found proteins are linked to the cytoskeleton (β-actin, myosin, tubulin) or to metabolism (e.g. glyceraldehyde 3-phosphate dehydrogenase, GAPDH). In agreement to their role as antigen presenting vesicles, many exosomes contain major histocompatibility complex (MHC) class I and II molecules. Of interest, exosomes contain also protein involved in cell signaling pathways, like β-catenin, Wnt5b or the Notch ligand, Delta-like 4, and mediators of intercellular cell signaling, like IL-1β, TNF-α and TGF-β (159).

As for proteins, the lipid composition of exosomes is distinct from that of the cell of origin, but is anyway characteristic of the cell type. Lipid composition analyses have been performed of exosomes derived from hematopoietic cells (141), oligodendrocytes (93) and melanoma cells (160). Internal membranes of MVBs are enriched in lipids, such as lysobisphosphatidic acid (LBPA), which plays an important role in exosome biogenesis. Moreover, exosomal membranes are often enriched in lipids usually associated with lipid rafts, such as cholesterol, sphingolipids, ceramide and glycerosphingolpids with long and saturated fatty-acyl chains (149, 161).

A major breakthrough in the field has been the discovery that exosomes contain nucleic acids, such as mRNAs and microRNAs (162, 163). As mRNAs were also shown to be translated in target cells, this was the first evidence suggesting cell-to-cell communication by exosome-mediated transfer of genetic information. Exosome-transferred miRNAs were also suggested to be functional in target cells, as miRNAs from T-cell exosomes caused inhibition of target genes in dendritic cells (156).

Not all mRNAs present in a cell are included in exosomes, so there is a specific targeting of mRNAs into exosomes, whose mechanisms are unknown. Recently, it was also reported that neural cells (164) and myoblasts (165) release exosomes carrying mitochondrial DNA (mtDNA). The role

of mtDNA is unknown, although it has been suggested that it could reach the cytosol of the target cells and be imported into the mitochondria.

1.5.3. The role of exosomes in the tumor microenvironment

Exosomes have been associated with tumor promoting activities. Exosomes may have a bimodal role in cancer. They can manipulate the local and systemic environment to support cancer in growth and dissemination and may also program the immune system to elicit an anti-tumor response. Exosomes in tumors are heterogeneous and secreted by all the cells, and this leads to a network of interactions that are likely complex (166) (Figure 1.5.2).

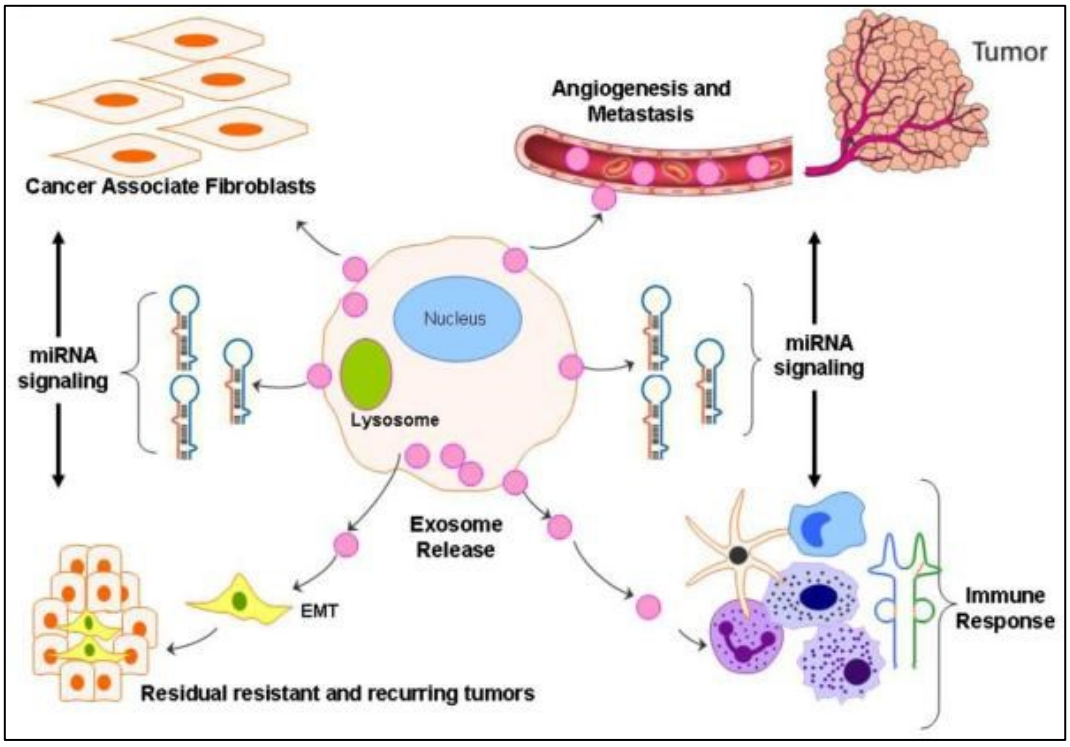

Figure 1.5.2. Role of exosomes in sustaining cancer resistance network. From: (167).

Al-Nedawi et al. demonstrated that tumor cells exchange proteins with oncogenic activity via exosomes' mediated transfer (168). A mutant epidermal growth factor receptor (known as EGFR vIII), residing on the exosome membrane derived from glioma cells, can be delivered to cells lacking this mutant form. The integration of EGFRvIII into these cells leads to an augmented expression of anti-apoptotic genes and an increase in anchorage-independent growth capacity.

Colon cancer cell harboring only mutant KRAS alleles are capable of releasing exosomes with mutant KRAS protein. Colon cancer cells expressing only the wild-type KRAS can internalize this mutant form. The exosomes' mediated exchange of mutant KRAS induces cell growth and tumorigenicity (169).

Tumor-derived exosomes can also modulate the tumor microenvironment and angiogenesis. For example, exosomes from prostate cancer cells and mesothelioma cell lines contain TGF-β1 protein, which is transferred to recipient cells in a biologically active form. *In vitro* experiments have shown that TGF-β1 expressing exosomes can trigger the differentiation of fibroblasts to myofibroblasts (170). Myofibroblasts are a key source of matrix-remodeling proteins within the tumor microenvironment and participate in tumor angiogenesis (171).

Recently, our group reported that exosome-mediate communication between cancer cells and stromal cell is bidirectional. In particular, mesenchymal stem cells isolated from human gliomas (called Glioma-Associated Stem Cells, GASC) can modulate the aggressiveness of glioblastoma initiating cells and glioblastoma cell lines by releasing exosomes (123).

Furthermore, cancer cells transfer membrane-bound EGFR to endothelial cells via exosomes. This transfer activates the autocrine VEGF/VEGFR-2 pathway in endothelial cells and likely support tumor angiogenesis (172). Moreover, melanoma-derived exosomes can deliver microRNA-9 (miR-9) into endothelial cells, promoting endothelial cell migration *in vitro* and tumor angiogenesis *in vivo* (173).

Several studies have shown that TD-exosomes can alter the extracellular matrix through secretion of matrix metalloproteinases (MMPs) or activators of MMPs, such as heat shock proteins. MMPs are zinc-dependent plasma membrane endo-peptidases that can degrade extracellular matrix proteins, such as collagen, fibronectin, proteoglycans and laminins. In fibrosarcoma and melanoma cells, it was shown that MT1-MMP was secreted in exosomes and could activate pro-MMP-2 and degrade collagen and gelatin (174). Other studies have demonstrated that heat shock proteins, such as hsp90, are also secreted via exosomes and can activate MMP-2 to enhance invasion of cancer cells (175).

Numerous reports indicate that exosome-mediated communication between tumor cells and the immune system is involved in recruiting pro-tumorigenic immune cells. For example, the blockade of exosome secretion by inhibiting Rab27a is associated with a decreased mobilization of neutrophils, which results in a reduced primary tumor growth and lung metastasis (176). Moreover, miRNAs in lung cancer released exosomes can silence the transcripts associated with Toll-like Receptor (TLR) family in macrophages. This mechanism stimulates macrophages to secrete pro-inflammatory cytokines, which support enhanced tumor dissemination (177). Cancer cells are also

able of inhibiting anti-tumor functions of the host immune system via an exosome-induced signaling. Chalmin et al. showed that tumor-derived exosomes activate myeloid-derived suppressor cells (MDSC), which exert immunosuppressive functions in cancer by suppressing the T-cell response (178, 179).

Tumor cells release large amount of microvesicles and the number of circulating microvesicles is increased in patients with cancer and correlates with poor prognosis (180). Thus, circulating exosomes may be potential biomarkers to predict cancer burden at an early stage and impact personalized cancer care. For example, a clinical subtype of glioblastoma contains EGFRvIII mutant/variant (163). The expression of this EGFRvIII mutant/variant is decisive in determining the therapeutic approach for such patients (181). In this regard, Skog and al. have demonstrated that circulating serum exosomes are positive for this mutant/variant EGFRvIII when the parental glioblastoma cells also expressed it (163). Therefore, determining EGFR status from a small serum sample derived exosomes instead of a need for primary tumor biopsy that involved invasive brain surgery, is of immense benefit for patients. Such application for circulating serum exosomes in tumor diagnosis have been described for colorectal cancer (182), ovarian cancer (183) and melanoma (184). Silva et al. have reported that high levels of exosomes in plasma of patients with colorectal cancer was significantly associated with poor differentiated tumors and with decreased overall survival (182). Patients with ovarian cancer exhibit significantly increased levels of serum exosomes compared to benign disease or healthy controls. A profile between exosome-derived miRNAs and corresponding tumor-derived miRNAs revealed a dysregulated expression of 3 out of 218 detectable miRNAs, suggesting a specific selection of miRNAs in tumor-derived exosomes (183).

Apart from serum, exosomes can be detected in further body fluid such as urine or saliva and provide non-invasive diagnostic markers for cancer (185, 186). Taken together, exosome-derived proteins and RNAs could serve as a useful tool to classify patients into different risk categories o enable a more tailored cancer therapy.

In addition, since cancer cells modify the local and systemic environment by releasing exosomes to support tumor progression and metastasis, impairing the secretion of exosomes by cancer cells may constitute a potential target for cancer therapy.

2. AIM OF THE STUDY

Gliomas are the most frequent tumors of the central nervous system. High-grade gliomas (HGG) are associated with disproportionately high morbidity and mortality and, despite an optimal treatment, the median survival is only 12 to 15 months for patients with glioblastoma multiforme. With respect to HGG, low-grade gliomas (LGG) grow slowly, but about 70% of grade II LGG evolve to anaplasia within 5-10 years (25).

Glioblastoma multiforme is the most common and malignant form of human glioma; it accounts for 60% of all glial tumors and occurs at a frequency of 5 cases per 100.000 people (187). Although molecular markers for glioblastoma multiforme have helped to identify patients responsive to current therapies (188), the overall survival of responsive patients has not changed substantially in the last two decades (189).

The aggressiveness of HGG is not only due to the high rate of tumor growth, but also to its particularly invasive nature, which allows the active egress of glioma cells from the tumor mass into the surrounding normal brain tissue (190). Therefore, invasive gliomas are essentially beyond the reach of current surgical therapies, which are localized to the tumor and immediate peri-tumor environment. However, glioblastoma multiforme cell invasion is not random; transformed glial cells preferentially invade along anatomic features such as myelinated axons, vascular basement membranes, and the subependyma (191), which suggest that they interact with specific structures in their immediate microenvironment.

Indeed, mounting evidence indicates that normal peritumoral cells plays active roles in tumor progression (98, 192) and a considerable interest is focused on the *tumor-associated stroma*. In fact, in addition to cancer cells, tumor lesions contain a mixture of different stromal cells, such as endothelial cells that constitute blood vessels, inflammatory cells that infiltrate the neoplastic tissue from the blood stream and resident stromal cells (95, 193). These stroma components have been studied essentially in solid tumors of epithelial origin, where Cancer Associated Fibroblasts (CAF) represent the most abundant cell type (194). Regarding gliomas, xenotransplantation experiments employing human glioma cell line have suggested that astrocytes in the vicinity can be activated and facilitate tumor invasiveness (102). The glial response to injuries, including the presence of a tumor mass, consists of multiple and complex changes in morphology, gene expression and function, a process referred to as “astrogliosis” (124). 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 (129, 133).

Thus, studying the tumor-stroma interplay could be an important opportunity for identifying key factors that mediate the activation of stromal cells toward a phenotype able to support tumor growth and molecular mechanisms driving tumor progression and anaplastic transformation. At this regard, recent studies suggest that the release of microvesicles could have an active role in communication processes occurring among different cell types populating the tumor microenvironment (163, 195, 196). However, little is known about this in gliomas.

In particular, our group has recently demonstrated that exosomes secreted by non-tumorigenic mesenchymal stromal cells isolated from human gliomas (Glioma-Associated Stem Cell, GASC) were able to increase the aggressiveness of glioma initiating stem cells *in vitro* (123). However, there is little information in the literature about the effects produced by glioma cells-derived microvesicles on the surrounding microenvironment.

Therefore, in order to shed light on communication mechanisms established between glioma cells and stromal cells within the tumor microenvironment, the work has been focused on the effects produced by exosomes released by different types of glioblastoma cells on astrocytes.

In particular, specific aims of this study were:

1. To verify if astrocytes grown in culture in the presence of exosomes deriving from a glioblastoma commercial cell line (U87-MG), primary isolated glioblastoma cells, and primary isolated glioblastoma stem cells, could acquire phenotypic and functional features of the reactive astrocytes populating the glioma microenvironment;
2. To verify whether exosomes produced by different glioblastoma cells could be able *in vitro* to induce a re-programming of glial cells by the acquisition of a stem cell potential;
3. To assess whether neural stem cells obtained by astrocytes conditioned with exosomes derived from glioblastoma cells could be differentiated in functional radial glia-like cells.

3. MATERIALS AND METHODS

3.1 Cells

Human glioblastoma 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. Glioblastomas were histopathologically evaluated by expert Pathologist following WHO criteria.

Glioblastoma stem cells (GSC) were isolated as described in (75, 123). Briefly, cells less than 40 μm in diameter were seeded at a density of $2x10^4$ cells/cm^2 onto laminin coated dishes in a growing medium composed as follows: Neurobasal-A medium (Gibco), 2 mM L-glutamine (Sigma-Aldrich), 1X N2 supplement (Gibco), 25 μg/mL Insulin, Penicillin-streptomycin (Sigma-Aldrich), 100 μg/mL human apo-trasferrin (Sigma-Aldrich), 1X B-27 supplement (Gibco), 20 ng/mL h-FGF-basic (Peprotech), 20 ng/mL h-EGF (Peprotech).GSC were cultured in an incubator at 37°C, 5%O2/5%CO2. Half of the medium was replaced with fresh one every 4 days. Once cells reached 70-80% of confluence, they were detached by TrypLE Express (Invitrogen) and re-plated at a density of 3-$4x10^3$/cm^2.

Glioblastoma primary cells (GBM) were isolated from glioblastoma samples and cultured in DMEM-low glucose (Gibco) supplemented with 10% fetal bovine serum (FBS; Euroclone) previously ultracentrifuged at 100,000 x g for 4 hours at 4°C (uFBS) and 1X Penicillin-streptomycin (Sigma-Aldrich).

Commercially available U87-MG glioblastoma cells (purchased from American Type Culture Collection) were cultured in DMEM-low glucose (Gibco) supplemented with 10% uFBS and 1X Penicillin-streptomycin (Sigma-Aldrich).

Commercially available Wi38 human normal lung fibroblasts (gently provided from Prof. C. Schneider) were cultured in DMEM-low glucose (Gibco) supplemented with 10% uFBS and 1X Penicillin-streptomycin (Sigma-Aldrich).

Cell culture supernatants from GSC (n=3), GBM (n=3), U87-MG and Wi38 cells were collected during the exponential growth phase for exosomes purification (see **3.2**).

Human normal astrocyte from cerebral cortex (ScienCell Research Laboratories) were cultured onto 0,1% poly-L-lysine (Sigma-Aldrich) coated-dishes in Astrocyte Medium (AM; ScienCell Research Laboratories) supplemented with 2% uFBS, 1X Astrocyte Growth Supplement (ScienCell Research Laboratories) and 1X Penicillin-streptomycin (ScienCell Research Laboratories) at 37°C, 5% CO_2.

3.2 Exosomes purification

Exosomes were purified from the collected cell culture supernatants by precipitation with ExoQuick-TC (System Biosciences), according to the manufacturer's protocol with minor modifications. Briefly, cell culture supernatants were harvested, centrifuged at 3,000 x g for 15 minutes to remove cells and cell debris and filtered through a 0,2 μm mesh filter to remove particles larger than 200 nm in diameter. Clarified supernatants were concentrated in their vesicular fraction by ultrafiltration with a centrifugal filter unit (cut-off 100 kDa, Amicon Ultra-15, Millipore) at 4,500 x g for 30 minutes. Concentrated supernatants were washed three times with D-PBS (Dulbecco's Phosphate Buffered Solution, Life Technologies) by ultrafiltration, mixed with ExoQuick-TC (5:1 v/v) and incubated *over-night* at 4°C. The mixture was centrifuged at 1,500 x g for 30 minutes and exosome pellet resuspended in 100 μL of D-PBS or dH_2O, according to downstream applications.
Exosomal protein yield was determined for each sample by Bradford assay (Bio-Rad).

3.3 Exosomes characterization

3.3.1 Dimensional analysis

Exosome dimension and concentration were assessed using a NanoSight LM10 instrument (NanoSight) in collaboration with Prof. Benedetta Bussolatti (University of Turin). NTA (Nanoparticle Track Analysis) software suite was utilized to calculate particles diameter and to construct the frequency distribution graph.

3.3.2 Flow cytometry

Purified exosomes were incubated with 4 μm aldehyde/sulfate latex beads (Invitrogen, Molecular Probes) in 1 ml of PBS and left shaking over night at room temperature. Solution were then added with 300 mL of 1 M glycine (Sigma-Aldrich) and, after 30 minutes, washed twice with PBS

containing 0.5% bovine serum albumin (Sigma-Aldrich) (PBS/BSA 0.5%). Exosomes conjugated with beads were finally incubated 20 minutes at room temperature with PE-labeled antibodies directed against CD63 and CD9 tetraspanins (Becton Dickinson). After two washes with PBS/BSA 0.5%, pellets obtained were re-suspended in 500 µL of PBS, and acquired by FACSCalibur and analyzed by Summit software (Beckman Coulter). PE-isotype-matched antibodies were used as a negative control.

3.4 Exosomes uptake by astrocytes

The interaction between astrocytes and exosomes secreted by glioma cells was investigated by evaluating the ability of astrocytes to internalize GSC-deriving exosomes. Briefly, 100 µg of purified exosomes resuspended in D-PBS were labeled with 5 µM Vybrant-DiD (Invitrogen) for 30 minutes at 37°C. Labeled exosomes (exo-DiD) were purified by precipitation with ExoQuick-TC (see **3.2**) and resuspended in Astrocyte Medium (AM) at the concentration of 10 µg/mL.
Astrocytes were seeded onto 0,1% poly-L-lysine coated-coverslips (Corning) and cultured in AM. Upon reaching 70-80% cell confluence, the culture medium was replaced with fresh new AM containing exo-DiD and cells incubated for 4 hours at 37°C/5% CO_2. Astrocytes conditioned by exo-DiD were fixed in 4% buffered paraformaldehyde (PFA, Sigma-Aldrich) for 20 minutes at *room temperature* (RT) and permeabilized with 0,1% Triton X-100 (Sigma-Aldrich) in PBS for 15 minutes at RT. Primary antibody incubation was performed for 2 hours at 37°C to detect *early-endosome antigen 1* (EEA-1; Abcam, 1:100), while Rhodamine Phalloidin (Molecular Probe, Invitrogen, 1:400) has been incubated for 1 hour at 37°C to detect actin filaments. To detect the primary antibody, A488-labeled secondary antibody (Molecular Probe, Invitrogen, 1:800) was employed. Vectashield (Vector) added with 1 µg/mL DAPI (Sigma-Aldrich) was used as mounting medium. Epifluorescence images were obtained utilizing a live cell imaging dedicated system consisting of a Leica DMI6000B microscope connected to a Leica DFC350FX camera (Leica Microsystems) equipped with a 5X objective (numerical aperture: 0.12) or a 40X oil immersion objective (numerical aperture: 1.25). Adobe Photoshop software was utilized to compose, overlay the images and to adjust contrast (Adobe).

3.5 Conditioning media and cultures

Exosomes purified from GSC (n=3), GBM (n=3), U87-MG and Wi38 cell culture supernatants were resuspended in AM supplemented with 2% uFBS (uAM) at the concentration of 10 µg/mL.
Human astrocytes were seeded onto 0,1% poly-L-lysine coated 6-cm Petri dishes at a cell density of 5,000 cells/cm^2 and cultured in the presence of the different conditioned uAM (3 mL/6-cm Petri dish) for 5 days at 37°C/5% CO_2. Cell culture supernatant was replaced with fresh new conditioned medium every 3 days. When astrocytes reached 75-80% cell confluence, they were either morphologically analyzed (see 3.6) ore detached and assayed as described below (see 3.7-3.11). Astrocytes cultured in uAM not added with exosomes have been utilized as negative control.

3.6 Morphological analysis

Astrocytes, grown in the presence of Wi38-, U87-MG, GMB- and GSC-exosomes for 5 days, were incubated with FITC labeled lectin from *Triticum vulgaris* (Sigma-Aldrich, 1:1000) in uAM for 30 minutes at 37°C/5% CO_2. After incubation, cell culture medium was discarded, cells washed twice with Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich) and new fresh uAM was added. Astrocytes grown in uAM devoid of exosomes were utilized as a control. Epifluorescence images were obtained utilizing a live cell imaging dedicated system consisting of a Leica DMI6000B microscope connected to a Leica DFC350FX camera (Leica Microsystems) and equipped with a 10X objective (numerical aperture: 0.25). The cell surface, the number of processes per cell and the length of the processes have been performed by Image-J.

3.7 Growth curve

Astrocytes deriving from the different experimental conditions (+Wi38-, +U87-MG-, +GBM-, and +GSC-exosomes) and from the negative control were plated at a cell density of 1,000 cells/cm^2 into 96-well BD high-content imaging plates (BD Falcon) and cell number was determined after staining of nuclei with 0,5 µM Hoechst for 20 minutes at 37°C. Cell culture supernatant was replaced with fresh new medium every 3 days. Cells were counted at day 0, 1, 2, 3, 4, 5, 6 and 7 after plating.

Triplicate wells were used at each time point. Population doubling time was calculated during the log-phase of the growth.

3.8 Scratch assay

Astrocytes conditioned or not by Wi38-, U87-MG-, GBM- and GSC-derived exosomes were plated at a cell density of 5,000 cells/cm^2 into a 6-well plate. At confluence, the cell monolayer was scraped in a straight line with a p10 pipette tip. Cellular debris was removed by washing with HBSS (Sigma-Aldrich) and the culture medium was replaced with fresh one. Images of at least 10 "scratches" for experimental conditions were acquired, at time intervals of 4 hours, by a Leica DMI 6000B microscope connected to a Leica DFC350FX camera (5X objective, numerical aperture: 0.12). Images were then compared and quantified by Image-J in order to calculate the rate of cell migration (197).

3.9 Soft agar assay

The ability of astrocytes (conditioned or not by Wi38-, U87-MG-, GBM- and GSC-derived exosomes) to grow in an anchorage-independent way has been evaluated by the soft agar assay. Briefly, 5x10^4 cells cultured in the different experimental conditions were resuspended in 2 mL of 0,25% agar supplemented with complete uAM or conditioned uAM and seeded in triplicates in 6-well plate containing a 1% agar base layer. Plates were incubated at 37°C in a humid atmosphere of 5% CO_2 for four weeks, with periodic additions of 1 mL of (conditioned) medium. To evaluate the adhesion independent growth of astrocytes conditioned or not by exosomes, soft agars were analyzed 1 day and 1, 2, 3 and 4 weeks after cell seeding. Specifically, a specific volume of the soft agar was analyzed acquiring a z-stack of phase contrast images with a live cell imaging dedicated system consisting of a Leica DMI 6000B microscope connected to a Leica DFC350FX camera (10X objective, numerical aperture 0.25). Once obtained, by using the LAS-AF software (Leica Microsystems), the sum image of the phase contrast channel, the number of seeded cells (day 1) and the number of colonies with a diameter greater than 60 microns (week 4) were determined. For each

replicate, a volume of 500μm^3 has been sampled. Results were expressed as number of colonies per 1x10^{3}seeded cells and colony diameter (μm).

3.10 Reverse transcriptase quantitative PCR analysis

Total RNA was extracted from astrocytes conditioned for 5 days by Wi38-, U87-MG-, GBM- and GSC-exosomes using the TRIzol Reagent (Invitrogen). Total RNA extracted from unconditioned astrocytes was used as a control. Genomic DNA was removed through DNaseI digestion (Qiagen). Total RNA was reverse-transcribed into cDNA using a SuperScript III cDNA synthesis kit (Invitrogen). The cDNA products were used for quantitative real-time PCR with the ready-to-use SYBR Green Master Mix gene expression kit (Bio-Rad). Real-time PCR was performed in 96-well plates with a LightCycler 480 system (Roche). The PCR conditions were as follow: 95°C for 3 minutes; 45 cycles at 95°C for 10 seconds, 60°C for 20 seconds and 72°C for 60 seconds. Primer pairs were the following: GFAP (glial fibrillary acidic protein) NCBI reference sequence NM_002055.4 primer FW 5'GGCAGAAGCTCCAGGATGAA3' and primer RW 5'CAGGGTGGCTTCATCTGCTT3' (product length 89 bp); nestin NCBI reference sequence NM_006617.1 FW 5'CTCAGCTTTCAGGACCCCAAG3' and primer RW 5'AGCAAAGATCCAAGACGCCG3' (product length 74 bp); vimentin NBCI reference sequence NM_003380 primer FW 5'GGCTCGTCACCTTCGTGAAT3' and primer RW 5'GCAGAGAAATCCTGCTCTCCT3' (product length 113 bp); GAPDH (glyceraldehyde-3-phosphate dehydrogenase) NCBI reference sequence NM_001256799.1 primer FW 5'GTCTCCTCTGACTTCAACAGCG3' and primer RW 5'ACCACCCTGTTGCTGTAGCCAA3' (product length 131 bp). Gene expression changes were calculated using a ΔΔCt method with statistical analysis (198).

3.11 Neurosphere formation assay

The ability of astrocytes (conditioned or not by Wi38-, U87-MG-, GBM- and GSC-derived exosomes) to grow as spherical aggregates in neural stem cell medium has been evaluated by the

neurosphere formation assay (199). Briefly, cells from each experimental condition were detached and resuspended at the concentration of $2x10^4$ cells/mL in neural stem cell medium containing Neurobasal-A medium (Gibco), 2 mM L-glutamine (Sigma-Aldrich), 1X B-27 supplement (Gibco), 20 ng/mL h-FGF-basic (Peprotech), 20 ng/mL h-EGF (Peprotech). 500 µL of cell suspension ($1x10^4$ cells) were seeded into a 24-well plate. Plates were incubated at 37°C in a humid atmosphere of 5% CO_2/5% O_2 for 1 week. To evaluate the formation of neurospheres, phase contrast images were acquired with a live cell imaging dedicated system consisting of a Leica DMI 6000B microscope connected to a Leica DFC350FX camera (5X objective, numerical aperture 0.12). The number of neurospheres with a diameter greater than 60 µm and the dimension of neurospheres were determined by Image-J.

Furthermore, in order to determine the ability of self-renewal, the neurospheres formed after 1 week were disaggregated in a single cell suspension, resuspended in neural stem cell medium at the concentration of $2x10^4$ cells/mL and 500 µL seeded into a MW-24. This operation was repeated once a week for a total of 4 weeks. The number of neurospheres with a diameter greater than 60 µm and the dimension of neurospheres were determined every 7 days by Image-J. Three independent experiments were performed in triplicates and analyzed. Results were expressed as number of neurospheres per $1x10^3$ seeded cells and as spheroid diameter (µm).

3.12 Induction of in vitro neural differentiation

Neurospheres obtained as in **3.11** were differentiated and analyzed as in (200). Briefly, neurospheres were dissociatated in a single cell suspension and $3x10^3$ cells/cm^2 were plated in DMEM high-glucose (Invitrogen), 10% FBS (Euroclone). After 24 h, the medium was replaced with DMEM high-glucose (Invitrogen), 10% FBS (Euroclone), 1X B-27 Supplement (Gibco), 10 ng/ml EGF (Peprotech EC) and 20 ng/ml bFGF (Peprotech EC). After 5 days, cells were washed and incubated for 5-10 days in DMEM containing 5 µg/ml insulin, 200 µM indomethacin and 0.5 mM IBMX (all from Sigma-Aldrich), in the absence of FBS. At the end of the treatment, cells were fixed with 4% buffered Paraformaldehyde, and assessed for the expression of neuronal (β3-tubulin, MAP-2), glial (GFAP) and oligodendroglial (O4) markers. The number of positive cells for the differentiation markers was determined.

3.13 Radial glia cell outgrowth assay

To assess the ability of neurospheres derived from exosome-conditioned and unconditioned astrocytes to give rise, *in vitro*, to elongations typical of radial glia cells, neurospheres obtained as in **3.11** were allow settling for 15-20 minutes in a Falcon tube. The cell culture medium was removed and individual neurospheres (100-200 μm in diameter) were resuspended in radial glia differentiation medium containing Neurobasal-A medium (Gibco), 2 mM L-glutamine (Sigma-Aldrich), 1X B-27 supplement (Gibco), 20 ng/mL h-EGF (Peprotech EC). Neurospheres were plated at the density of 100 spheres/cm^2 onto 0,1% poly-L-lysine (Sigma Aldrich) coated-48-well plate and incubated at 37°C in a humid atmosphere of 5% CO_2/5% O_2 (80). Neurospheres were cultured for additional 3 days to allow the formation of radial processes between the spheres. Triplicates of each experimental condition were performed. To evaluate the formation of radial glia cell (RGC) processes, phase contrast images were acquired with a live cell imaging dedicated system consisting of a Leica DMI 6000B microscope connected to a Leica DFC350FX camera (10X objective, numerical aperture 0.25). Results were expressed as percentage of spheres extending RGC processes.

3.14 Migration of newborn neurons along the radial process

To assess for the presence of migrating neurons, cells on the radial processes obtained as in **3.13** were observed over time by taking photograph every 10 min for 1 h using a Leica DMI 6000B inverted fluorescence microscope connected to a Leica DFC350FX camera (40X objective, numerical aperture 0.65).

3.15 Immunofluorescence

Astrocytes deriving from the different experimental conditions, neurospheres grown either in expansion or differentiation medium and radial glia cells were fixed in 4% buffered paraformaldehyde for 20 minutes at RT. For intracellular staining, fixed cells were permeabilized

for 10 minutes at RT with 0,1% Triton X-100 (Sigma-Aldrich) before exposing them to primary antibodies. Primary antibody incubation was performed *over-night* at 4°C to detect: CD133 (Milteny, 1:100), CD44 (NeoMarkers, 1:100), FABP-7 (Cell Signaling, 1:1000), GD3 (Abcam, 1:100), GFAP (Dako, 1:50), Ki67 (Leica, 1:100), MAP-2 (Millipore, 1:1000), N-Cadherin (Dako, 1:100), Nestin (Millipore, 1:100), NeuN (Millipore, 1:100), Numb (Cell Signaling, 1.100), O4 (Millipore, 1:100), Sox-2 (Millipore, 1:500), s100β (Abcam, 1:100), Tenascin C (Abcam, 1:10), β3-tubulin (Tuj1, Abcam, 1:1000), Vimentin (Dako, 1:50). To detect primary antibodies, A488 and A555 dyes labeled secondary antibodies, diluted 1:800, were employed (Molecular Probe, Invitrogen). Finally, Vectashield (Vector) added with 1 μg/ml DAPI (Sigma-Aldrich) was used as mounting medium. Epifluorescence 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) and equipped with a 63X oil immersion objective (numerical aperture: 1.40), a 40X oil immersion objective (numerical aperture: 1.25), a 20X objective (numerical aperture: 1.00) or a 10X objective (numerical aperture: 0.25) Adobe Photoshop software was utilized to compose, overlay the images and to adjust contrast (Adobe).

3.16 Statistics

Characteristics of the study population are described using means ± s.d. or median and 25^{th}-75^{th} percentiles for continuous variables and percentages for categorical variables. Data were tested for normal distribution using the Kolmogorov-Smirnov test. T-test or Mann-Whitney test, as appropriate, was used to compare continuous variables between two groups. Effects of exosomes and supernatant on different cell types were performed, as appropriate, by repeated measurements one-way Anova followed by Bonferroni post-test (Prism, version 4.0c) or Kruskal-Wallis followed by Dunn's post-test. P values less than 0.05 were considered significant.

4. RESULTS

4.1 GSC release exosomes that are internalized by astrocytes

In order to establish whether Glioblatoma Stem Cells (n=3) release exosomes in the supernatants, these latter were collected during the exponential growth phase and exosomes were precipitated by ExoQuick-TC (System Biosciences). The presence of exosomes was confirmed both by FACS (presence of microviscicles expressing the specific exosomal protein markers CD9 and CD63, Figure 4.1.1A) and by NanoSight (presence of particles with a mean diameter of about 200 nm, Figure 4.1.1B).

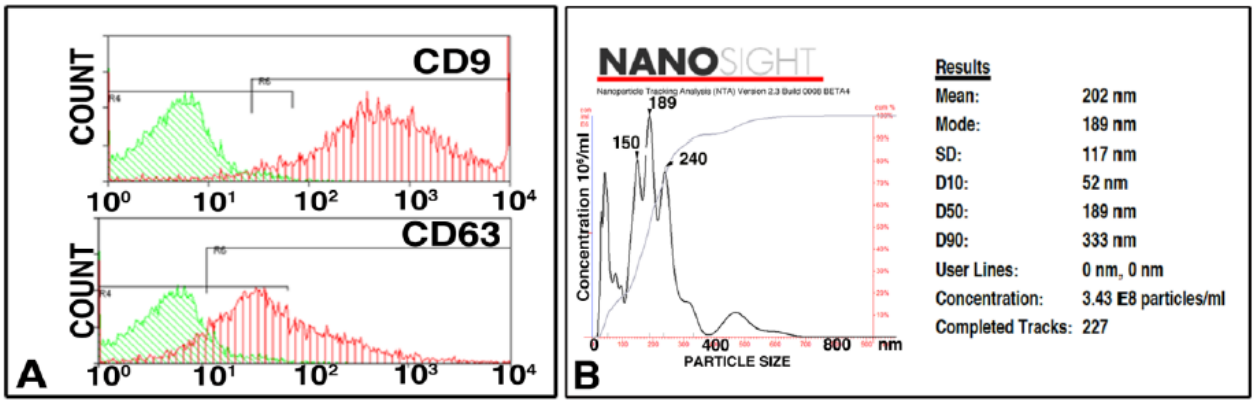

Figure 4.1.1 (A) FACS analysis of GSC-derived exosomes. Histograms overlays show isotype control IgG staining profile (green histograms) versus specific antibody staining profile (red histograms). (B) NanoSight analysis show the size distribution profile of GSC-derived exosomes.

In order to establish whether normal glial cells could interact with exosomes secreted by glioma cells, astrocytes were incubated for 4 hours with DiD-labeled GSC-exosomes and cells were examined by epifluorescence microscope. Since one of the mechanisms by which cells internalize exosomes seems to be mediated by the formation of endocytic vesicles (201), we assessed whether exosomes produced by GSC and internalized by astrocytes were able to co-localize with endosomes. As shown in Figure 4.1.2A, DiD-labeled exosomes could be identified within the cells and partially co-localized with endosomes, identified by EEA-1 (Figure 4.1.2B). The internalization of DiD-labeled exosomes was further confirmed by FACS (Figure 4.1.2C).

Same results were obtained analyzing the supernatant obtained from Wi38, GBM and the glioblastoma cell line U87, confirming that microvesicles purified from these cell types express exosomal markers CD9 and CD63 and are able to be internalized by astrocytes (data not shown).

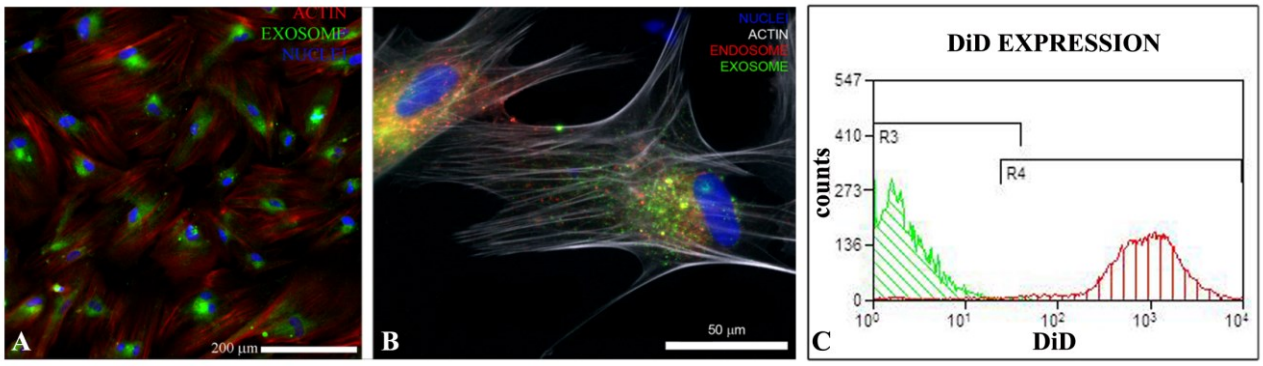

Figure 4.1.2. (A-B) DiD-labeled exosomes (green fluorescence) are internalized by astrocytes, identified by actin (red fluorescence in A. and white fluorescence in B.) and endosomes (red fluorescence in B.). Nuclei are depicted by the blue fluorescence of DAPI. (C) Histograms overlay showing astrocytes cultured (red histogram) or not (green histogram) with DiD-labeled exosomes.

4.2 Exosomes released by glioma cells induce in normal human astrocytes an activated phenotype

Reactive astrocytes are characterized by morphological changes (augmented cell size and ramifications), increased proliferation and migration ability and by the expression or over-expression of specific proteins.

In order to assess the biological effect of exosomes produced by different cell types of glioma cells on normal astrocytes, these latter were grown in culture media conditioned for 5 days by primary glioblastoma cell cultures (GBM), glioma stem cells (GSC) and the commercially available U87 glioblastoma cell line. As controls, we cultured astrocytes either in a medium conditioned by exosomes produced by normal fetal fibroblasts (Wi38) or in an un-conditioned medium. Conditioned and unconditioned astrocytes were then analyzed in terms of morphology, biological behavior (growth kinetic, migration ability, anchorage-independent growth) and expression of molecular markers distinguishing the phenotype of "reactive astrocyte".

Changes in cell morphology were assessed evaluating cell size, number of primary processes per cell soma and length of processes. When grown in the presence of U87-MG-, GBM- and GSC-exosomes, astrocytes showed a significant increase in both cell area (Figure 4.2.1F) and length of the cellular processes (Figure 4.2.1H) compared to Wi38-conditioned and unconditioned cells. Furthermore, only astrocytes grown in the presence of exosomes produced by primary glioma

cultured cells showed a higher number of primary processes extending from a single cell soma (Figure 4.2.1G).

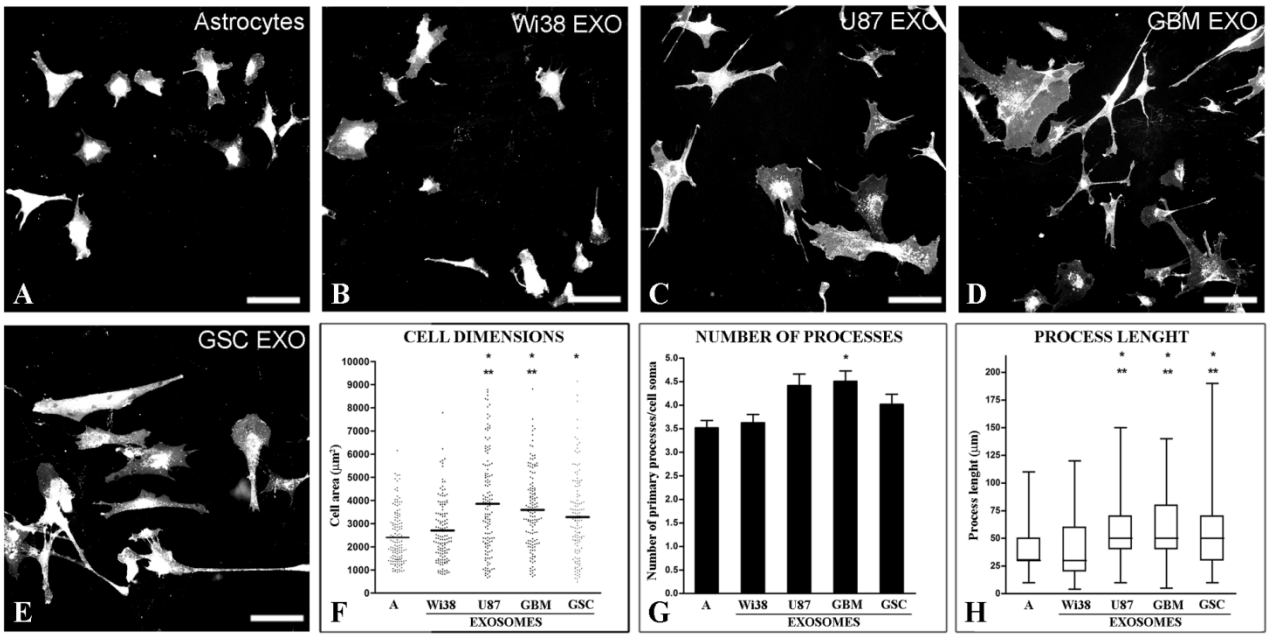

Figure 4.2.1. Morphological analysis of exosome-conditioned astrocytes. (A-E) Fluorescence microscopy images of lectin-FITC labeled astrocytes (A) conditioned by Wi38-exo (B), U87-MG-exo (C), GBM-exo (D) and GSC-exo (E). Scale bar 50 µm. (F) Scatter dot plot representing the distribution of measured values. (G) Results are expressed as mean ± standard deviation. (H) Box and whiskers plot representing the distribution of measured values. *, p<0.05 vs astrocytes (A); **, p<0.05 vs Wi38 exosomes.

The ability of exosomes produced by different cell types of glioma cells to modulate the proliferative capacity of astrocytes was evaluated comparing the growth kinetics of these latter in the different experimental conditions. As shown in Figure 4.2.2H, exosome released by glioma cells were able to significantly increase the proliferation rate of astrocytes compared to control conditions, being the population doubling time 31±2 h for U87-MG-exo and GBM-exo , 30±2 h for GSC-exo, 34±3 h for WI38-exo and 35±3 h for unconditioned cells, respectively. Moreover, as shown in Figure 4.2.2A-F, a significant increase in the number of cells positive for the proliferation marker Ki67 was observed when glial cells were grown in the presence of exosomes produced by U87-MG (66±3%), GBM (67±4%) and GSC (72±6%) compared to unconditioned astrocytes (54±5%) or astrocytes conditioned by Wi38-exosomes (55±7%), confirming the notion that microvesicles released by glioma cells are able to stimulate the proliferation of glial cells.

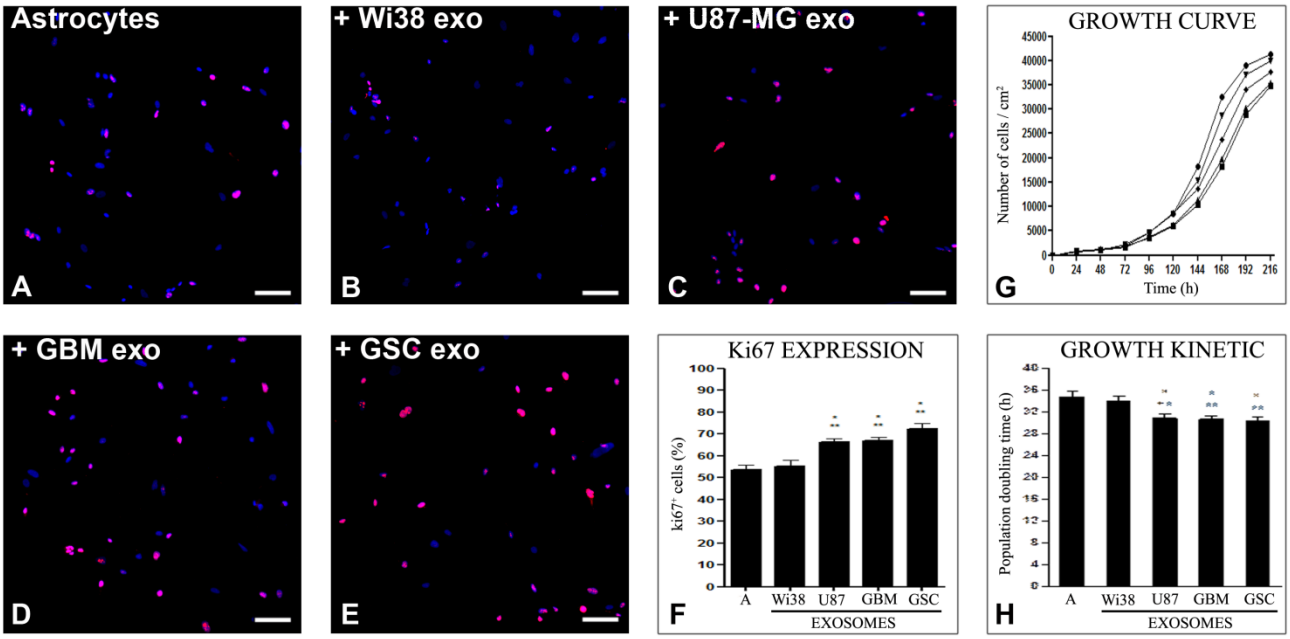

Figure 4.2.2. Growth kinetics. (A-E) Nuclear expression of Ki76 (red fluorescence) in unconditioned (A) and astrocytes conditioned by Wi38 exosomes (B), U87-MG exosomes (C), GBM exosomes (D) and GSC exosomes (E). Nuclei are depicted by the blue fluorescence of DAPI. Scale bar 100 µm. (F, H) Results are expressed as mean ± standard deviation. (G) ■ astrocytes, ▲ Wi38 exo, ▼ U87-MG exo, ◆ GBM exo, ● GSC exo. *, p<0.05 vs astrocytes (A); **, p<0.05 vs Wi38 exo.

In order to verify whether the migration ability of astrocytes could be modified by the presence of exosomes, a scratch assay was performed and the migration rate of glial cell in each experimental condition was calculated. In all the different conditions, cells were able to close the wound in 24 hours. However, as shown in Figure 4.2.3C, glial cells conditioned by exosomes deriving from U87-MG, GBM and GSC were characterized by an increase in the migration rate with respect to glial cells unconditioned or conditioned by WI38-exosome during the first 12 h, even though only astrocytes grown in the presence of exosomes released by GSC were characterized by a significantly higher migration rate compared to control conditions and were able to partially close the wound after just 12 h (Figure 4.2.3A). Interestingly, analyzing the migration speed in the individual time intervals (0h-4h, 4h-8h, 8h-12h), a different reactivity of the cells in the different experimental conditions was observed. In particular, astrocytes conditioned by exosomes produced by glioma cells (U87, GBM and GSC) were about 2 times faster during the first 4 hours, compared to the other cells ($p<0.05$ for U87 and GBM, and $p<0,01$ for GSC). However, over time the migration speed decreased at the same level of control cells at the time point of 12 h (Figure 4.2.3D).

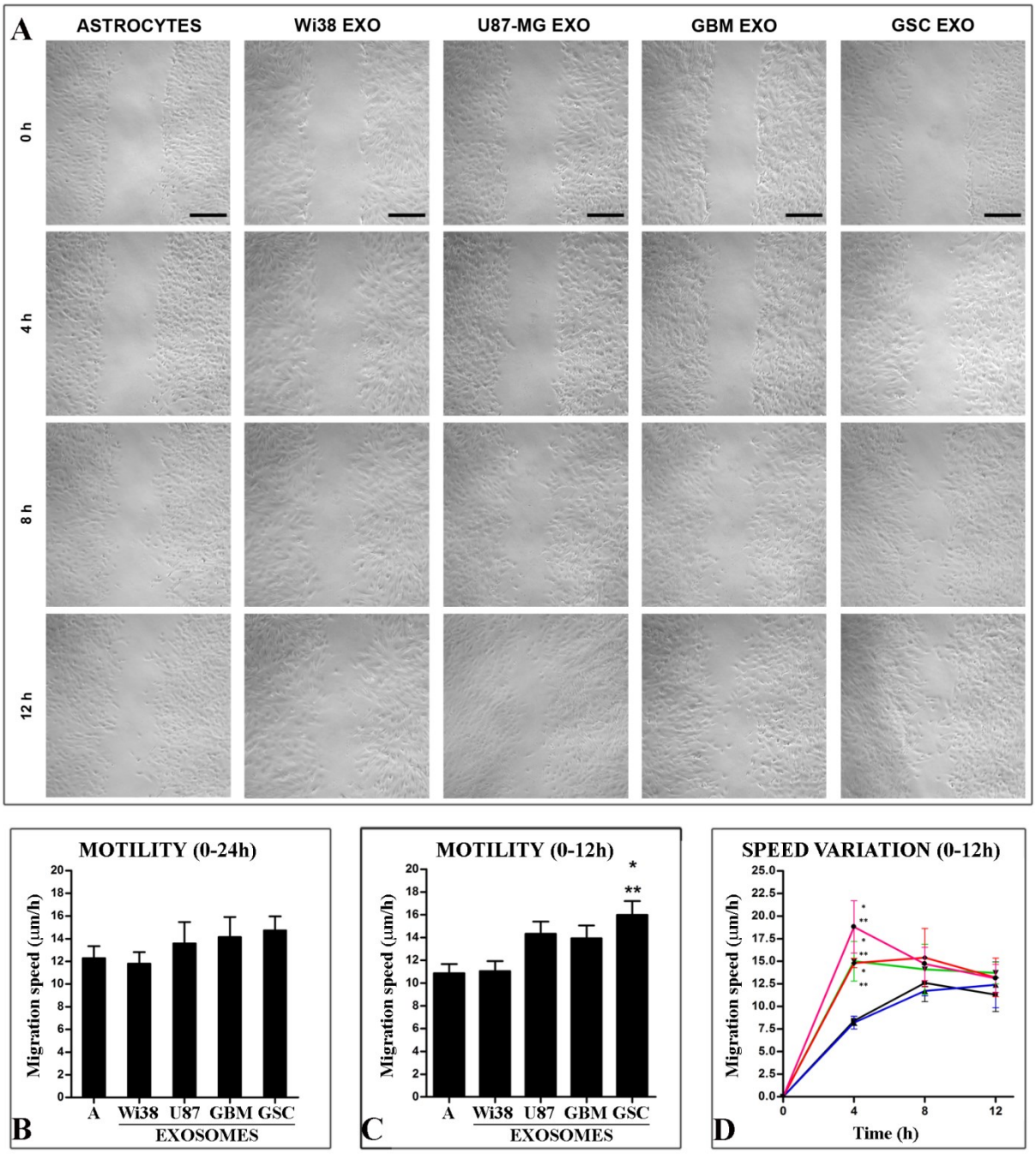

Figure 4.2.3. Cell motility (scratch assay). (A) Representative images, taken at different time points, of astrocytes grown in the different experimental conditions. Scale ba 500 µm. (B) Results are expressed as mean ± standard deviation. (C) Migration speed at the different time points for each experimental condition: astrocytes (black), +Wi38 exo (blue), +U87-MG exo (green), +GBM exo (red), +GSC exo (purple).
***, p<0.05 vs astrocytes (A); **, p<0.05 vs Wi38 exosomes.**

The ability of exosomes released by U87 cells, GBM cells and GSC to induce aberrant growth features in normal glial cells was investigated evaluating the ability of conditioned astrocytes to grow in the absence of cell adhesion. 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 (202).

As shown in Figure 4.2.4, when grown for 4 weeks in semisolid agar medium and in the presence of exosomes produced by U87 (4.2.4C), GBM cells (4.2.4D) and GSC (4.2.4E), astrocytes acquired the ability, compared to unconditioned glial cells (4.2.4A), to form about 67% more colonies that were 22% larger in diameter.

Conversely, exosomes deriving from Wi38 cells did not affect the anchorage-independent growth ability of astrocytes (4.2.4B).

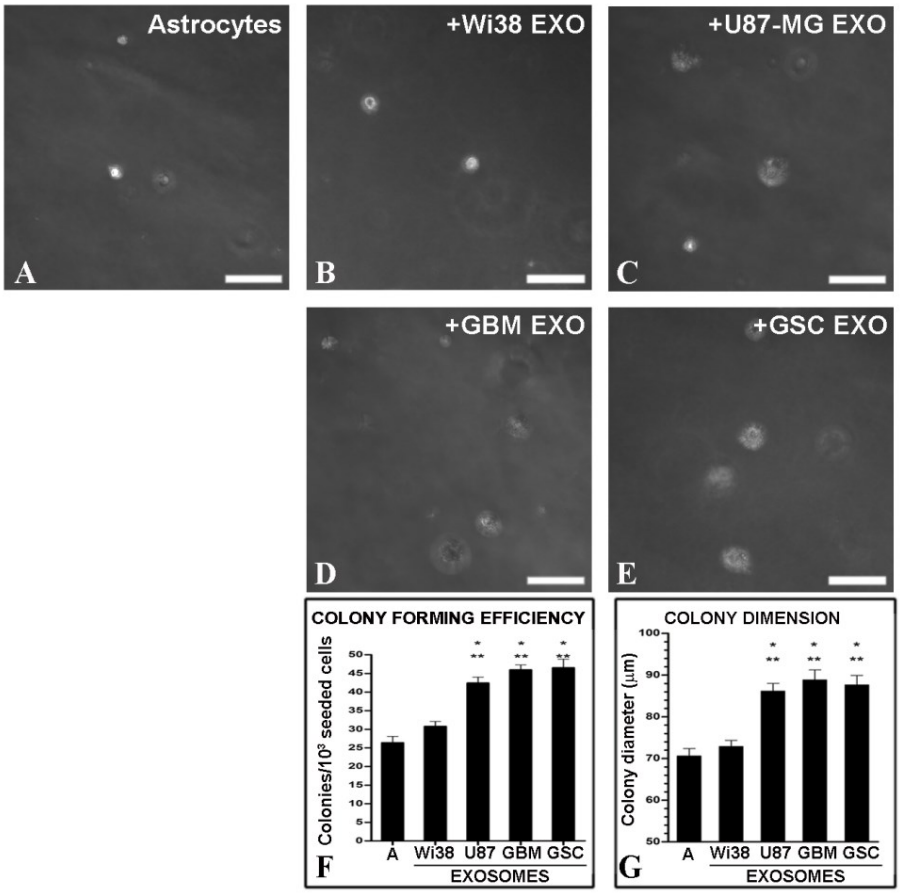

Figure 4.2.4. Anchorage-independent growth of astrocytes (soft agar assay). (A-E) Phase contrast images of colonies formed by unconditioned (A) and astrocytes conditioned by exosomes released by Wi38 (B), U87-MG (C), GBM (D) and GSC (E). Scale bar 500 µm. (F-G) Results are expressed as mean ± standard deviation.
***, p<0.05 vs astrocytes (A); **, p<0.05 vs Wi38 exosomes.**

In order to verify whether alterations in the biological behavior of astrocytes conditioned by exosomes released by glioblastoma cells were paralleled by modifications in the expression of proteins and mRNAs characterizing reactive astrocytes, these latter were assessed both by immunofluorescence (4.2.5A) and quantitative real-time PCR (4.2.5C). In this regard, as shown in Figure 4.2.5B, exosomes secreted by different cell types exerted variable effects on protein expression, while, as expected and in line with results previously obtained, vesicles secreted by Wi38 cells did not appreciably modify the expression of the tested proteins. Microvesicles produced

by U87, GBM and GSC shared the ability to significantly increase the percentage of astrocytes expressing CD44 (89.8%, 95.6% and 93.5%, respectively) and GD3 (51.9%, 43.3% and 52.6%, respectively), compared to control astrocytes (74% for CD44 and 25.9% for GD3). However, while exosomes secreted by U87 and GBM showed a substantially comparable effect to modulate the expression of all the tested markers, exosomes produced by GSC were the only ones able to both reduce the fraction of cells positive for GFAP (-14.4% compared to control cells and -30% compared to U87-exo and GBM-exo) and significantly increase the number of astrocytes expressing vimentin (+17.6% compared to control cells), indicating the appearance of a fraction of astrocytes less differentiated. Accordingly, GSC-exosomes increased the number of glial cells positive for proteins whose expression is correlated to a neural stem-like phenotype such as nestin (50.3% compared to 16.4% of unconditioned cells; 3.6-fold increase in mRNA levels) and FABP-7 (35.3% compared to 7.7% of control astrocytes). Also U87-derived microvesicles significantly increased the expression of nestin compared to unconditioned cells, but to a lesser extent than GSC-derived microvesicles, whereas GBM-microvesicles did not. In both cases, levels of nestin mRNA were twice the control. Interestingly, the effect of GSC-exosomes to promote the expression FABP-7 in glial cells was observed neither in U87-exo (12%), nor in GBM-exo (17%).

Altogether, the results obtained indicate that glioblastoma cells, both derived from a commercial cell line or primary cultured cells, are capable of inducing the activation of astrocytes by means of exosomes release. Indeed, after being grown in the presence of these latter, astrocytes acquire morphological, functional and phenotypic features distinguishing the phenotype of reactive astrocyte.

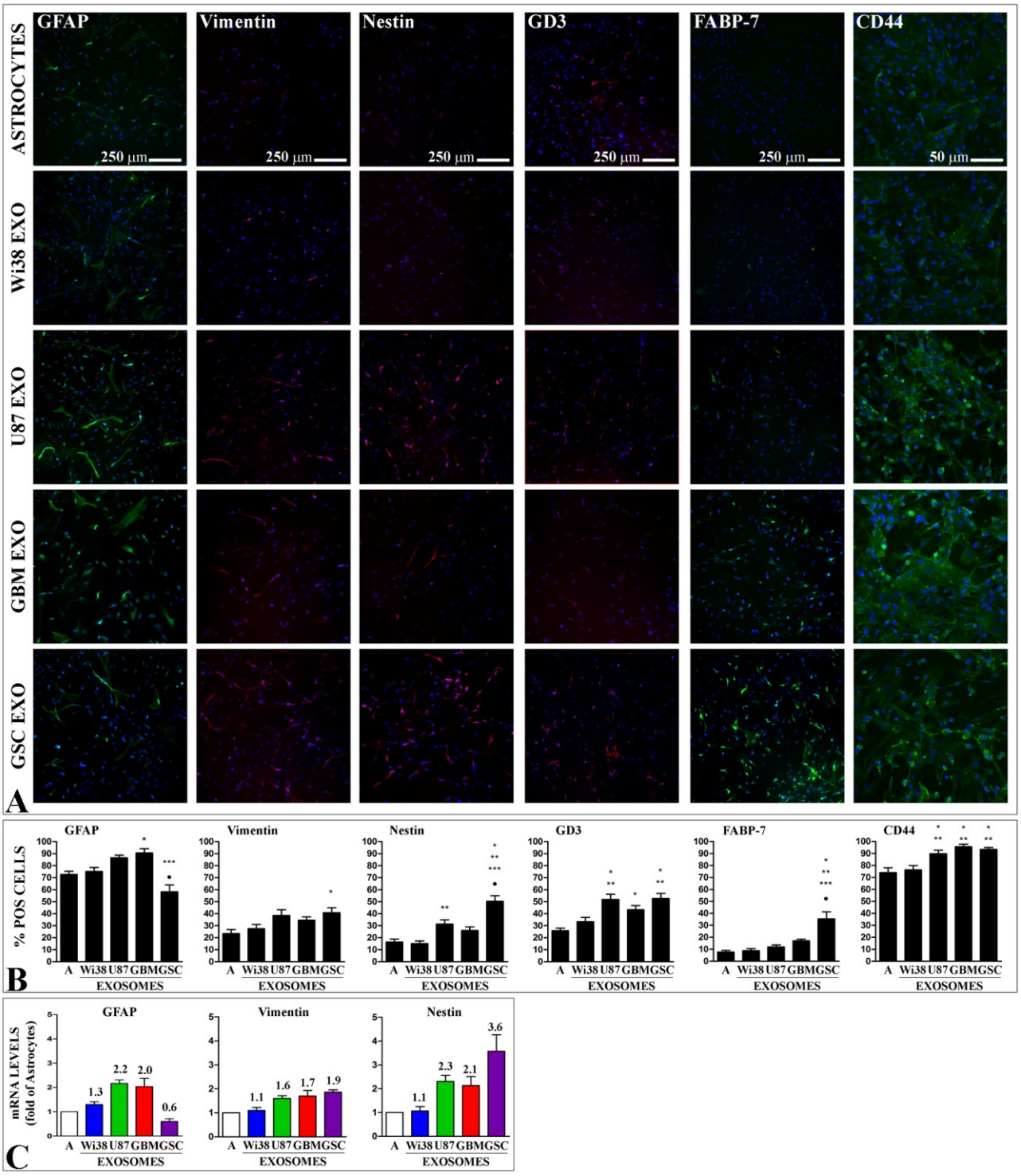

Figure 4.2.5. Expression of activation markers. (A) Representative fluorescence images of GFAP, vimentin, nestin, GD3, FABP-7 and CD44 in unconditioned astrocytes and in astrocytes conditioned by Wi38-, U87, GBM and GSC-derived exosomes. Nuclei are depicted by the blue fluorescence of DAPI. (B) Results are expressed as mean ± standard deviation. (C) mRNA expression levels of GFAP, vimentin and nestin determined by q-RT-PCR. Data are presented as mean fold increase in exosomes-conditioned cells compared to unconditioned cells ± standard deviation. Values above bars indicate fold change in exosomes-conditioned astrocytes.

***, p<0.05 vs astrocytes (A); **, p<0.05 vs Wi38 exosomes; ***, p<0.05 vs U87 exosomes; ●, p<0.05 vs GBM astrocytes.**

4.3 Only exosomes released by GSC promote the acquisition of a neural stem-like potential by astrocytes

In vivo studies in animal models have demonstrated that astrocyte activation is often associated with cellular reprogramming and acquisition of stem cell potential (124, 131-133). Therefore, we assessed the ability of exosomes released by different types of glioblastoma cells (commercial cell line U87-MG, primary gliobastoma cells GBM and primary gliobastoma stem-like cells GSC) to mediate the dedifferentiation of normal astrocytes into neural stem-like cells.

Astrocytes conditioned for 5 days in the presence of exosomes isolated from Wi38, U87, GBM and GSC were detached and seeded in culture conditions selective for the growth of neural stem cells in suspension as neurospheres (203). After a week, number and size of the spherical aggregates growing in suspension produced by unconditioned and conditioned astrocytes were determined.

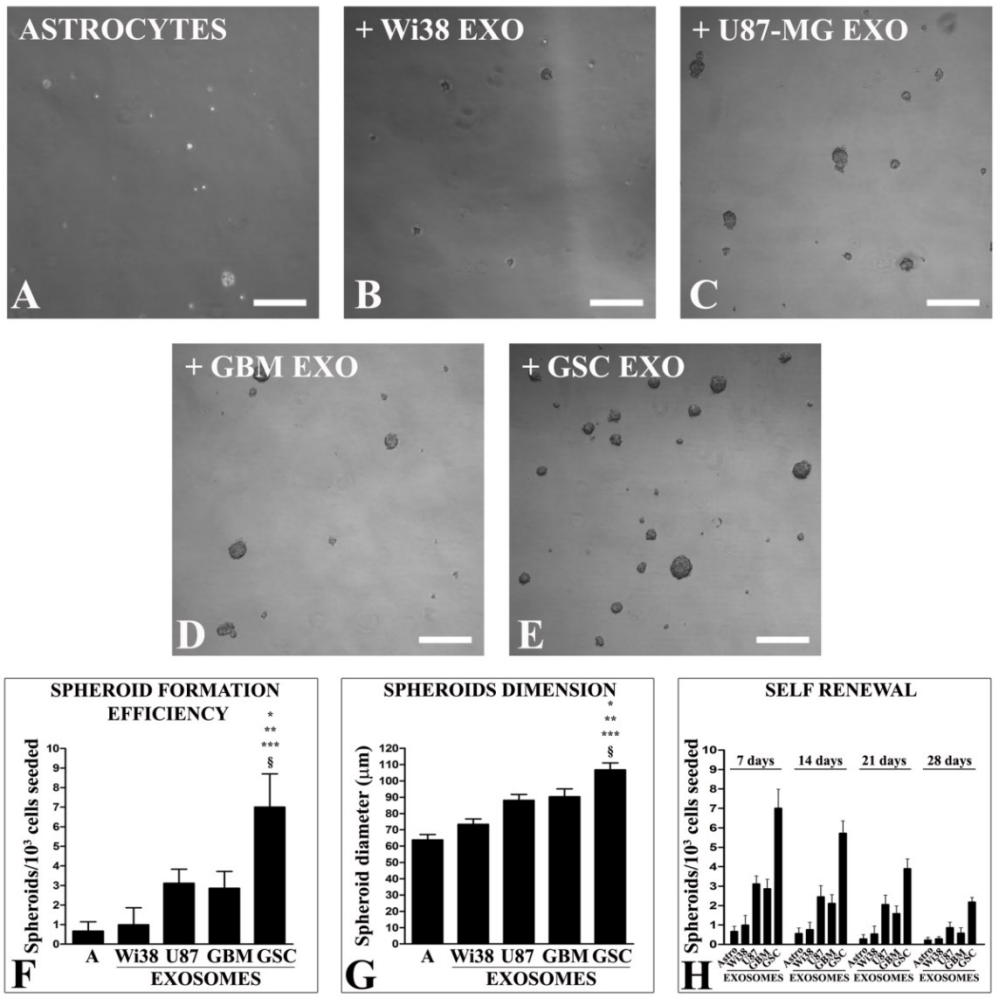

Figure 4.3.1 Neurosphere assay. (A-E) Representative phase contrast images of neurospheres generated by astrocytes unconditioned (A) and conditioned by exosomes produced by Wi38 (B), U87 (C), GBM (D) and GSC (E). Scale bar 500 µm. (F-H) Results are expressed as mean ± standard deviation.
***, p<0.05 vs astrocytes (A); **, p<0.05 vs Wi38 exosomes; ***, p<0.05 vs U87 exosomes; §, p<0.05 vs GBM exosomes.**

As shown in Figure 4.3.1F, astrocytes grown in the presence of microvesicles released by glioblastoma cells tend to form more neurospheres compared to control cells, however, the presence of spherical aggregates was significantly increased only in the culture of astrocytes previously conditioned with GSC-exosomes, characterized by a spheroid formation efficiency 10 times higher compared to unconditioned astrocytes. Moreover, as shown in Figure 4.3.1G, astrocytes treated with GSC-exosomes were able to generate neurospheres significantly bigger than all other conditions (+43% and +9% in diameter compared to unconditioned and glial cells conditioned by U87- and GBM-exosomes, respectively). Microvesicles released by Wi38 cells did not alter the spheroid formation efficiency of glial cells.

To establish whether spheroids were characterized by neural stem cell properties we evaluated their expression of stem cell markers, their self-renewal capacity and their multipotency.

Neurospheres obtained from astrocytes conditioned by GSC-exosomes (named "astrospheres") were characterized by the expression of protein markers distinguishing the neural stem cell phenotype. As shown in Figure 4.3.2, astrospheres expressed CD133 (A) and Sox-2 (B), particularly in the inner mass of the sphere, N-cadherin (C), the intermediate filaments nestin (D) and vimentin (E) and the radial glia cell marker FABP-7 (F).

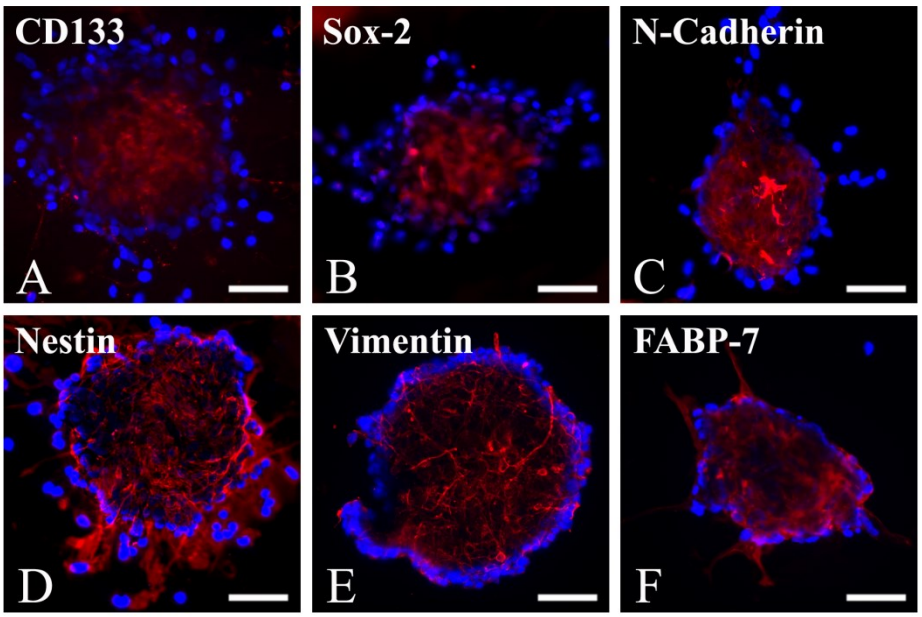

Figure 4.3.2. Fluorescence microscopy images of the expression of neural stem cell markers in neurospheres derived from astrocytes conditioned by GSC-exosomes. Red fluorescence represent the expression of CD133 (A), Sox-2 (B), N-cadherin (C), nestin (D), vimentin (E) and FABP-7 (F), respectively. Nuclei are depicted by the blue fluorescence of DAPI. Scale bar 50 µm.

To assess the self-renewal capacity of neurospheres generated by unconditioned and exosomes-conditioned astrocytes, spheroids were dissociated and single-cell suspensions re-plated every 7 days over a period of 4 weeks. As shown in Figure 4.3.1H, the spheroid-forming efficiency (SFE) of astrocytes unconditioned and conditioned by Wi38-exosomes was less than $1/10^3$ seeded cells over time. The SFE of astrocytes conditioned by U87- and GBM-exosomes was ~$3/10^3$ seeded cells after the first week, but decreased to <$1/10^3$ seeded cells at the fourth. Neurospheres generated by astrocytes grown in the presence of exosomes released by GSC were characterized by a SFE of $7/10^3$ seeded cells after the first 7 days and was the only one condition to maintain a SFE >$2/10^3$ seeded cells at the 4^{th} passage in culture (28 days).

In order to verify whether astrospheres were multipotent, spheroids obtained after 7 days in culture in neural stem cell medium were cultured under appropriate differentiation inducing conditions, and the acquisition of neuronal, glial and oligodendroglial markers was assessed (Figure 4.3.3).

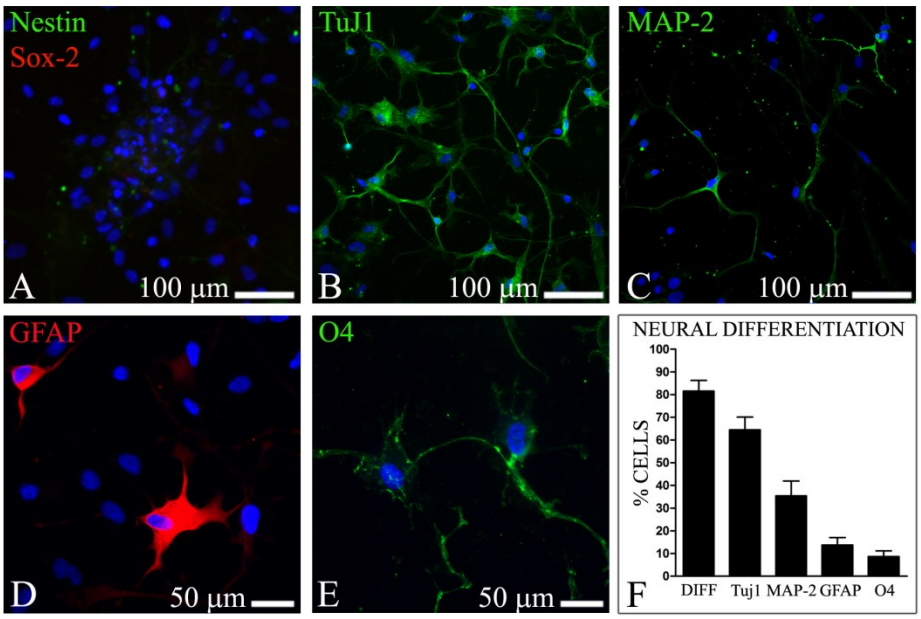

Figure 4.3.3. Multipotency of astrospheres. Nestin (green fluorescence, A), Sox-2 (red fluorescence, A), Neuronal Class III β-tubulin (identified by the Tuj1 antibody, green fluorescence, B), MAP-2 (green fluorescence, C), GFAP (red fluorescence, D) and O4 (green fluorescence, E) expression in differentiated cells. Nuclei are depicted by the blue fluorescence of DAPI staining. (F) Results are expressed as mean ± standard deviation.

Neurospheres exposed to neural differentiation medium lost the expression of neural stem-like markers Sox-2 and nestin, the presence of which were detectable only at the level of the inner mass of spheroids (Figure 4.3.3A). About 80% of the cells displayed the expression of differentiation markers (Figure 4.3.3.F). Specifically, more than 60% and more than 30% of the cells were positive for the neuronal markers β3-tubulin (identified by Tuj1 antibody) and MAP-2, respectively, while

about 10% of the cells were found to be positive for the specific glial marker GFAP. Interestingly, 5% of the cells also acquire the ability to differentiate into O4-positive oligodendrocyte-like cells.

In conclusion, exosomes released by glioblastoma stem cells (GSC) promote the de-differentiation of normal human astrocytes, increasing their growth efficiency as spherical aggregates in suspension. These neurospheres, here called “astrospheres”, possess neural stem cell-like features, being able to express neural stem cell markers and to differentiate into neuronal, glial and oligodendroglial lineages.

4.4 Astrospheres are able to differentiate into functional radial glia cells

Astrospheres could be expanded in neural stem cell medium for at least 4 passages in culture (12-16 population doublings), as shown in Figure 4.3.1H. However, spheroids were also able to adhere to the substrate showing a growth modality characterized by the establishment of a bipolar or unipolar morphology, by the formation of long and straight filaments that connected individual spheres (Figure 4.4.1A) and by the presence of migrating cells along the processes (Figure 4.4.1B-C). This growth pattern is described in the literature as characteristic of *in vitro* cultured radial glia cells (85, 204, 205). Therefore, in order to verify whether astrospheres were able to generate radial glia-like cells, adherent spheres were characterized in terms of morphology, phenotype and function.
Firstly, a radial glia cell (RGC) outgrowth assay was performed in order to assess the ability of spheroids derived from unconditioned and GSC-exosomes-conditioned astrocytes to give rise to radial glia-like processes. As shown in Figure 4.4.1G, after 3 days in adhesion onto poly-L-lysine coated-plates in EGF-containing medium, spheroids obtained from astrocytes conditioned by GSC-exosomes (E-F) gave rise to a radial glia-like culture, being able to extend radial-like processes among adjacent spheres with a significantly higher efficiency (62.5±8.2%) compared to those obtained from unconditioned glial cells (13.3±4.8%, respectively, $p<0.0001$, D).

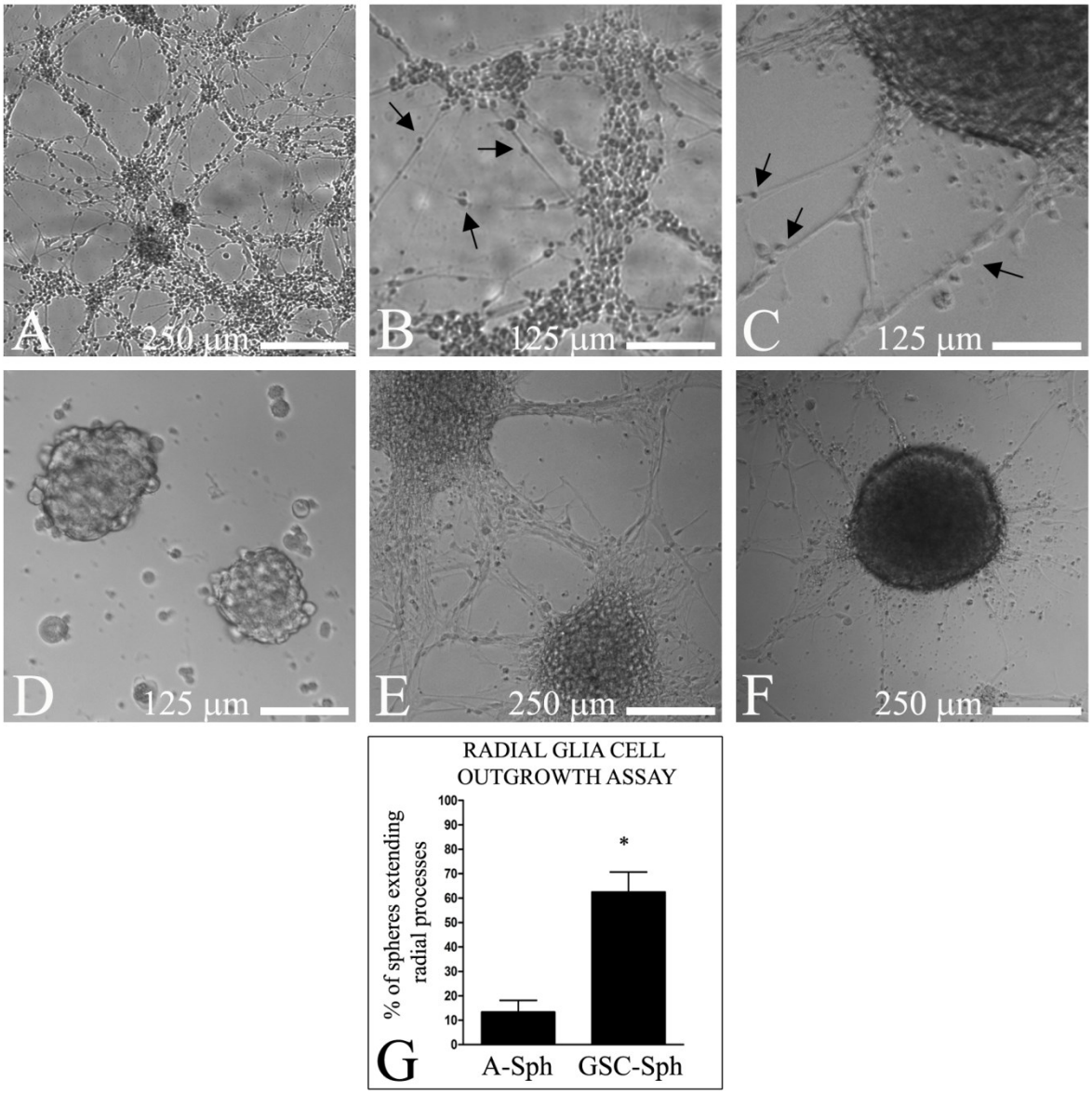

Figure 4.4.1. Astrospheres can differentiate in radial glia-like cells. (A) Growth pattern of adherent astrospheres 3 days after plating. (B, C) Magnifications showing the presence of cells associated to radial-like fibers (arrows). (D-F) Representative images of adherent spheroids deriving from unconditioned (A-Sph, D) and GSC-exosomes conditioned astrocytes (GSC-Sph, E, F). (G) Results are expressed as mean ± standard deviation. *, p<0.0001 vs A-Sph.

Astrospheres extending radial glia-like fibers were then subjected to immuno-staining in order to verify whether the long thin filaments were indeed RGC processes rather than axons. Therefore, the expression of neuronal versus RGC markers was examined. As shown in Figure 4.4.2A1-3 and B1-3, the elongated processes that extended between astrospheres co-expressed the RGC markers FABP-7, nestin and vimentin. Moreover, FABP-7-positive filaments did not co-express neuronal markers β3-tubulin (Figure 4.4.2C) and NeuN (Figure 4.4.2D). Interestingly, as can be observed in the magnified images C1-3, the expression of β3-tubulin identified neuronal filaments associated to RGC fibers, but distinct from them. RGC are characterized by a neurogenic potential *in vitro* and *in vivo* (206-208), being able to undergo an asymmetric division giving arise to a RG cells and to an

immature neuron-committed cell able to migrate on the RGC fiber. Therefore, the ability of RGC to perform asymmetric divisions was tested. As shown in Figure 4.4.2E-F, a fraction of cells were positive for both the expression of β3-tubulin and the marker of asymmetric division Numb but not for nestin, suggesting that astrosphere-derived RGC were able to perform asymmetric divisions in order to give rise to a neuronal-committed progeny.

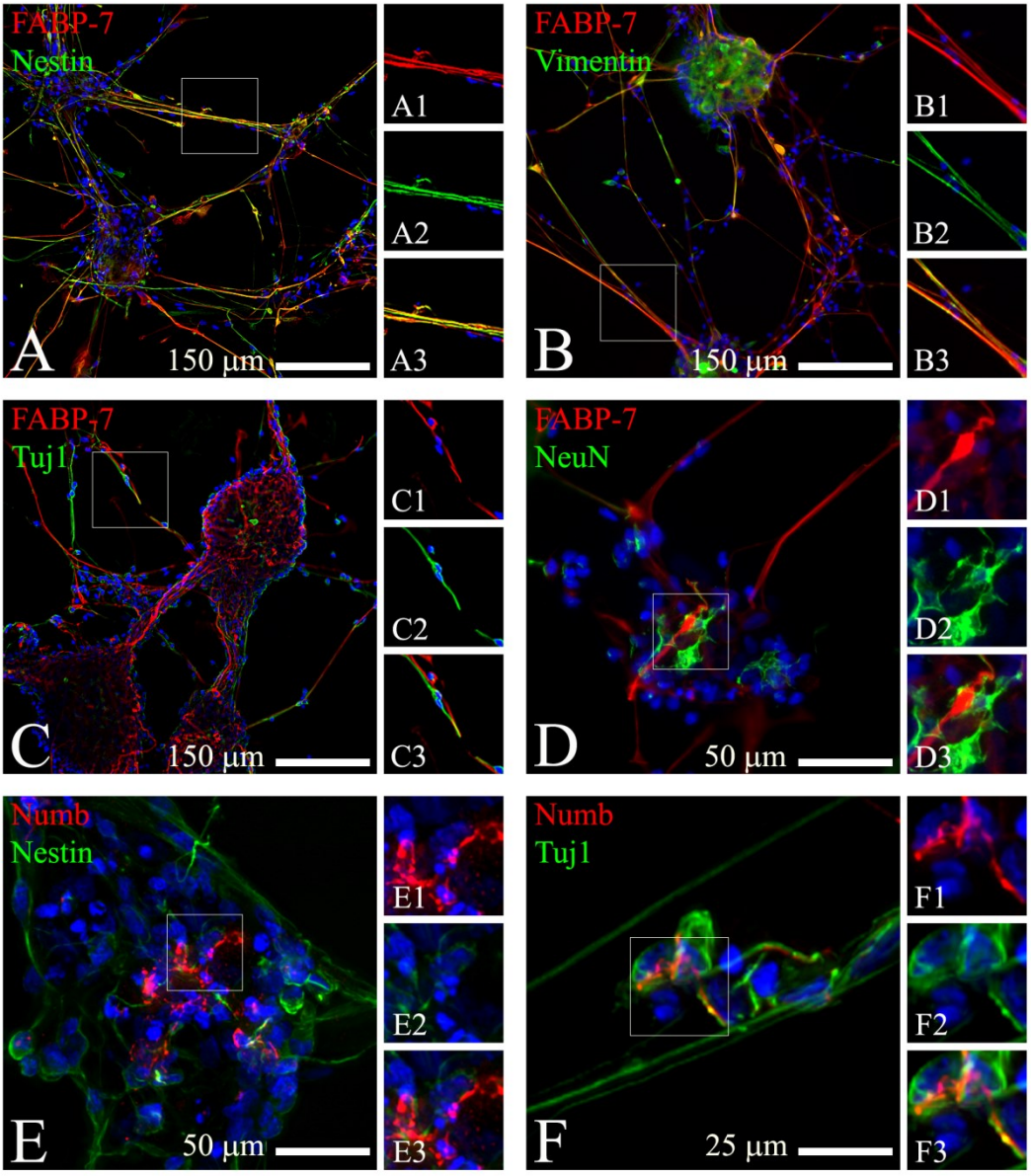

Figure 4.4.2. Phenotypic characterization of astrosphere-derived radial glia cells. (A, A1-3) Expression of FABP-7 (red fluorescence, A1) and nestin (green fluorescence, A2). (B, B1-3) Expression of FABP-7 (red fluorescence, B1) and vimentin (green fluorescence, B2). (C, C1-3) Expression of FABP-7 (red fluorescence, C1) and β3-tubulin (identified by the Tuj1 antibody, green fluorescence, C2). (D, D1-3) Expression of FABP-7 (red fluorescence, D1) and NeuN (green fluorescence, D2). (E, E1-3) Expression of Numb (red fluorescence, E1) and nestin (green fluorescence, E2). (F, F1-3) Expression of Numb (red fluorescence, F1) and Tuj1 (green fluorescence, F2). Nuclei are depicted by the blue fluorescence of DAPI staining.

Therefore, in order to test whether RGC were functional by virtue of their ability to support migration of immature neurons on their processes, time-lapse microscopy was used to monitor cell movement along the radial fiber. As shown in Figure 4.4.3, cells attached to RGC fibers were indeed migrating (A) and were characterized by the morphology of motile neurons, with an elongated process extending in the direction of movement expressing of the neuronal marker Tuj1 in the absence of the co-expression of the RGC marker FABP-7 (B and C).

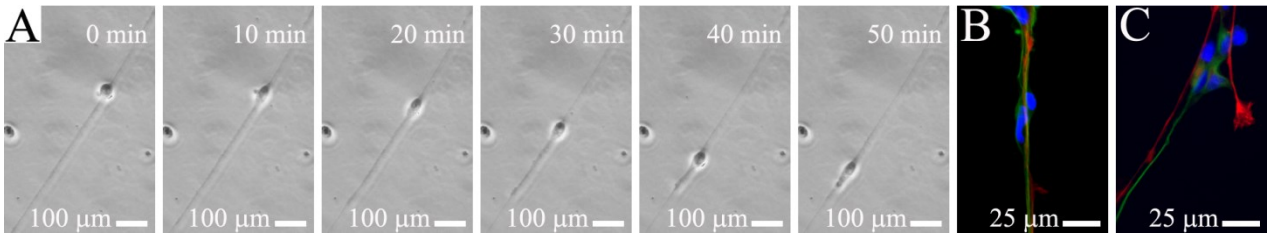

Figure 4.4.3. Migration of the neuronal-committed progeny of RGC along the radial process. (A) Representative time-lapse phase contrast microscopy of a cell attached to RGC fiber e migrating along it. Images acquisition was performed every 10 minutes for 50 minutes. (B-C) Representative fluorescence microscopy images of cells migrating along RGC processes for the expression of Tuj1 (green fluorescence) and FABP-7 (red fluorescence). Nuclei are depicted by the blue fluorescence of DAPI staining.

Therefore, these results suggested that neurospheres obtained from glial cells grown in culture in the presence of microvesicles released by glioblastoma stem cells were able to spontaneously differentiate into functional radial glia-like cells, characterized by the expression of both neuroepithelial and astroglial markers and by the ability to support the migration of immature neurons.

5. DISCUSSION

The importance of the microenvironment in tumor progression has been extensively reported in the last few years and the active role of microenvironment in supporting tumorigenesis, invasion and metastasis is widely recognized.

The tumor microenvironment in the brain consist of many different cell types that involve brain-resident and brain-infiltrating cells. In this context, endothelial cells that constitute the microvascular blood vessels, microglia-macrophages, and astrocytes have received the most attention so far. One of the current challenges in understanding the role of the tumor microenvironment is poor definition of different cell populations. For example, new phenotypes or subtypes of myeloid cells have been described in recent years, and further cellular subtypes are expected to emerge in the future. As for astrocytes, has been shown that they differ in various brain locations and their phenotype changes upon the exposure of different stimuli.

Astrocytes are glial cells that become activated in response to different central nervous system (CNS) injuries (124, 209-211). Astrocyte activation is known as reactive gliosis and is characterized by cellular hypertrophy and changes in gene expression patterns, including the up-regulation of the Glial Fibrillary Acidic Protein (GFAP) and the acquisition of stem-like potential (129, 133, 212). After a CNS insult, reactive astrocytes form a scar tissue surrounding the damaged area and this has been shown to be essential for the local containment of inflammation and for the repair of the demarcated tissue (212). Reactive astrocytes have been frequently observed in the vicinity of primary and metastatic brain tumors in animal model, as well in human patients (213, 214). *In vitro* studies demonstrated that a variety of factors released by astrocytes can support the growth of primary and metastatic brain tumor cells, including the secretion of substances that promote cancer cell proliferation and invasion, suppression of adaptive immune responses through the repression of microglia activation and induction of T-cell apoptosis, and protection of cancer cells from apoptosis through direct cell-cell interactions (215-218). However, little is known about molecular mechanisms responsible for astrocyte activation, in particular in the context of brain tumors, and the complex role of microenvironment in the glioma progression is still poor understood, as well as the interactions occurring between cancer cells and stromal cells in the brain.

A continuous cross-talk between glioma cells and stroma is required for the development of a permissive environment, able to promote the effective tumor growth and dissemination. Emerging evidence suggest that extracellular microvesicles released by cancer cells may play an important and active role in local and systemic cell-cell communication in cancer (219). In particular, a lot of

interest has been directed to exosomes (166). Exosomes are small membrane vesicles of endocytic origin secreted by most cell types. They can contain a large number of biologically active molecules, such as proteins, mRNAs, microRNAs and DNA fragments, and are shuttled from a donor cell to a recipient cell (162, 163). Cancer-derived exosomes may contribute to the recruitment and reprogramming of the tumor microenvironment to form a pro-tumorigenic and permissive soil able to stimulate tumor growth and dissemination and by eliciting an anti-tumor response. Several studies in the last decade demonstrated that exosomes can mediate the transfer of oncogenic proteins between cancer cells, modulate the tumor microenvironment by stromal cells activation, extracellular matrix remodeling and angiogenesis stimulation, and the immune system response against tumor antigens (168-170, 173). Recently, our group has identified a novel class of mesenchymal stromal cell in human gliomas. This cells, although devoid genetic alterations of the tumor of origin, are characterized by stem cell properties, aberrant growth features, a gene expression profile typical of tumor-supporting cells and are able to enhance *in vitro* the aggressiveness of glioblastoma initiating stem cells by means of exosome release, thus supporting the notion that microvesicles released by stromal activated cells can have an active role in tumor progression (123).

Therefore, since the mechanisms that mediate glial cells activation in response to the presence of a tumor mass have not yet been characterized, we decided to verify if the release of microvesicles by glioma cells could represent a key factor able to promote astrocyte activation. In particular, in order to take into account the heterogeneity of tumor cells and to evaluate whether different glioma cell models could induce different outcomes, we compare the effects produced on glial cells by exosomes obtained from a commercial cell line of gliobastoma (U87-MG) and from primary cultured glioblastoma cells (GBM) and glioblastoma stem cells (GSC).

Numerous experimental evidences suggest that microvesicles released by glioblastoma cells are able to support tumor growth by means of angiogenesis stimulation and the formation of a hypoxic microenvironment (163, 220, 221). However, studies regarding microvesicles released by glioblastoma stem cells are not yet been reported. Therefore, we first demonstrated that the latters are able to secrete exosomes. In analogy to what reported in literature, exosomes purified from GSC tend to be larger than those produced by non-cancer cells (222). In fact, vesicles purified from culture supernatants of GSC possess an average size of about 190 nm in diameter and expressed tetraspanins CD9 and CD63, commonly recognized exosomal markers. Furthermore, GSC-exosomes can be internalized by normal human astrocytes and internalized vesicles partially co-localize with endosomes, thus suggesting an uptake modality mediated by the formation of endocytic vesicles.

Astrocytes grown in the presence of exosomes purified from different cell types of glioma cells assume morphological and phenotypical characteristics of reactive astrocytes. In fact, only after 5 days in conditioned culture medium, glial cells are characterized by an increased cell size and number and dimension of cellular processes. Furthermore, exosome-conditioned astrocytes showed an increased proliferation and migration rate and acquired the ability to form colonies in semisolid culture medium, an aberrant growth feature shared by transformed and tumor-associated cells (202). These alterations of the biological properties of glial cells were also associated with modifications of astrocyte phenotype. Exosome-conditioned astrocytes were characterized by an increased expression of GFAP, vimentin, GD3 and CD44. These results are in agreement with what has been observed about the reactive astrocyte phenotype (209, 212, 223, 224). As demonstrated by Katz, CD44 and tenascin-C are expressed in glioblastoma tissues at a higher level in the perivascular astrocytes when compared to peritumoral astrocytes, suggesting subtle differences between the two populations of non-neoplastic tumor-associated cells (225). Interestingly, exosomes purified from different types of glioblastoma cells produced different effects on glial cells. Indeed, while microvesicles obtained from U87 and GBM cells showed a very similar behavior in stimulating an activated phenotype of astrocytes, exosomes derived from GSC were the only ones able to decrease the expression of GFAP and, in the same time, increase nestin and FABP- 7 levels in glial cells, two proteins that characterize neural stem cell and radial glia phenotypes, respectively (226, 227).

In the adult cerebral cortex mature astrocytes lack expression of nestin and vimentin, decrease GFAP levels and do not proliferate. However, astrocytes exposed to injury return to express these proteins, proliferate and may resume properties of glia present at earlier developmental stages (127, 228, 229). Therefore, we decided to verify whether exosomes secreted by glioblastoma cells were able to mediate de-differentiation effects on glial cells. Among all the experimental conditions tested, only exosomes produced by GSC were able to significantly increase the propensity of astrocytes to grow as spheroids in neural stem cell medium. Neurospheres obtained possessed self-renewal ability, expressed neural stem cell markers CD133, Sox-2, nestin, vimentin and N-cadherin and could be induced to differentiate *in vitro* in neurons, astrocytes and oligodendrocytes. These results are very important because: 1. provide a mechanism by which glioblastoma cells can modulate the behavior of glial cells by inducing a “reactive-astrocyte-like” phenotype through the release of microvesicles, and 2. represent the first experimental evidence of the fact that exosomes can act as mediators of cellular de-differentiation processes. Yang et al. has recently demonstrated that β-catenin signaling could be the molecular event that initiate astrocyte activation, and that the dysregulation of the β-catenin signaling pathway could be responsible for the transformation of normal glial cells and the formation of an astrocytoma (230). However, leading events that induce

astrocyte activation in brain tumors still remain unclear, as well as relationships existing between activated stromal cells and tumor cells in gliomas.

Furthermore, neurospheres obtained by GSC-exosomes-conditioned astrocytes (which we called "astrospheres"), once let adhere, showed a greater ability to give rise to cultures of functional radial glia cells (RGC) compared to those obtained from normal unconditioned astrocytes. RGC were characterized by the presence of long and straight filaments extending between adjacent spheres and by the expression of FABP-7, nestin and vimentin, and were able to support the migration of immature newborn neuronal-committed cells along the radial process. In fact, these migrating cells expressed neuronal markers in the absence of the expression of radial glia markers. During brain development, FABP-7 is expressed in radial glial cells, where it plays a role in the establishment and maintenance of the radial glial fiber system that guides immature migrating neurons to their final destination (82, 231). Radial glial cells give rise to GFAP-expressing astrocytes once neuronal migration is completed and can also display properties of precursor cells, generating both neurons and glial cells *in vitro (232)*. Moreover, a role as neural progenitors or neural stem cell has been proposed for radial glial cells (233). Interestingly, the radial glia marker FABP-7 is expressed by a subset of malignant glioma cell lines established from high-grade astrocytomas, and it's associated with an increased cellular motility and invasive ability. In human glioma tissues, FABP-7 expression is correlated with patient's prognosis (83, 234). However, although the expression markers of radial glia markers in gliomas has so far been associated only to tumor cells, the results obtained in this work show that also glial cells could re-express neural stem cells- or radial glial cell-features when they grow in the presence of exosomes released by GSC.

In conclusion, in this work we propose a mechanism for astrocyte activation in the context of brain tumors based on the release of microvesicles by glioblastoma cells. In addition, we demonstrate that exosomes secreted by glioblastoma initiating stem cells possess a potent effect on the acquisition of stem-like features by glial cells, which can also spontaneously differentiate in functional radial glia, a particular phenotype characteristic of the earlier developmental stages, whose expression is correlated with a migratory and invasive phenotype of glioma cells. Taken together, these results suggest that microvesicles released by glioblastoma stem cells possess a molecular content able to profoundly modify the homeostasis of brain tissue inducing cell reprogramming processes and contributing to anaplasia and heterogeneity of the neoplastic tissue. Studying the molecular cargo of exosomes could be important to understand the tumor biology, by the identification of molecular factors which may possess a key role in activating stromal cells toward a tumor-supporting phenotype.

6. REFERENCES

1. Theele DP & Streit WJ (1993) A chronicle of microglial ontogeny. *Glia* 7(1):5-8.

2. Lewis PD (1968) The fate of the subependymal cell in the adult rat brain, with a note on the origin of microglia. *Brain : a journal of neurology* 91(4):721-736.

3. Neuhaus J & Fedoroff S (1994) Development of microglia in mouse neopallial cell cultures. *Glia* 11(1):11-17.

4. Perry VH & Gordon S (1988) Macrophages and microglia in the nervous system. *Trends in neurosciences* 11(6):273-277.

5. Ling EA & Wong WC (1993) The origin and nature of ramified and amoeboid microglia: a historical review and current concepts. *Glia* 7(1):9-18.

6. Eglitis MA & Mezey E (1997) Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. *Proceedings of the National Academy of Sciences of the United States of America* 94(8):4080-4085.

7. Foran DR & Peterson AC (1992) Myelin acquisition in the central nervous system of the mouse revealed by an MBP-Lac Z transgene. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 12(12):4890-4897.

8. Del Rio-Hortega P (2012) Studies on neuroglia: Glia with very few processes (oligodendroglia) by PA-o del RA-o-Hortega. 1921. *Clinical neuropathology* 31(6):440-459.

9. Dawson MR, Levine JM, & Reynolds R (2000) NG2-expressing cells in the central nervous system: are they oligodendroglial progenitors? *Journal of neuroscience research* 61(5):471-479.

10. Bradl M & Lassmann H (2010) Oligodendrocytes: biology and pathology. *Acta neuropathologica* 119(1):37-53.

11. Baumann N & Pham-Dinh D (2001) Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiological reviews* 81(2):871-927.

12. Lunn KF, Baas PW, & Duncan ID (1997) Microtubule organization and stability in the oligodendrocyte. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 17(13):4921-4932.

13. Roussel G, Delaunoy JP, Nussbaum JL, & Mandel P (1979) Demonstration of a specific localization of carbonic anhydrase C in the glial cells of rat CNS by an immunohistochemical method. *Brain research* 160(1):47-55.

14. Kaur C, Hao AJ, Wu CH, & Ling EA (2001) Origin of microglia. *Microscopy research and technique* 54(1):2-9.

15. Sugita Y, Tokunaga O, Terasaki M, Morimatsu M, & Shigemori M (2003) Epithelial differentiation in medulloblastoma: comparison with other embryonal tumors of neuroectodermal origin. *Pathology international* 53(12):858-864.

16. Ohgaki H (2009) Epidemiology of brain tumors. *Methods in molecular biology* 472:323-342.

17. Ohgaki H & Kleihues P (2005) Epidemiology and etiology of gliomas. *Acta neuropathologica* 109(1):93-108.

18. Louis DN, *et al.* (2007) The 2007 WHO classification of tumours of the central nervous system. *Acta neuropathologica* 114(2):97-109.

19. Jiang Y & Uhrbom L (2012) On the origin of glioma. *Upsala journal of medical sciences* 117(2):113-121.

20. Lindberg N, Kastemar M, Olofsson T, Smits A, & Uhrbom L (2009) Oligodendrocyte progenitor cells can act as cell of origin for experimental glioma. *Oncogene* 28(23):2266-2275.

21. Furnari FB, *et al.* (2007) Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes & development* 21(21):2683-2710.

22. Weiler M & Wick W (2012) Molecular predictors of outcome in low-grade glioma. *Current opinion in neurology* 25(6):767-773.

23. Pouratian N & Schiff D (2010) Management of low-grade glioma. *Current neurology and neuroscience reports* 10(3):224-231.

24. Bourne TD & Schiff D (2010) Update on molecular findings, management and outcome in low-grade gliomas. *Nature reviews. Neurology* 6(12):695-701.

25. Soffietti R, *et al.* (2010) Guidelines on management of low-grade gliomas: report of an EFNS-EANO Task Force. *European journal of neurology : the official journal of the European Federation of Neurological Societies* 17(9):1124-1133.

26. Cancer Genome Atlas Research N (2008) Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 455(7216):1061-1068.

27. Mischel PS, *et al.* (2003) Identification of molecular subtypes of glioblastoma by gene expression profiling. *Oncogene* 22(15):2361-2373.

28. Louis DN (2006) Molecular pathology of malignant gliomas. *Annual review of pathology* 1:97-117.

29. Maher EA, *et al.* (2006) Marked genomic differences characterize primary and secondary glioblastoma subtypes and identify two distinct molecular and clinical secondary glioblastoma entities. *Cancer research* 66(23):11502-11513.

30. Phillips HS, *et al.* (2006) Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer cell* 9(3):157-173.

31. Parsons DW, *et al.* (2008) An integrated genomic analysis of human glioblastoma multiforme. *Science* 321(5897):1807-1812.

32. Ducray F, El Hallani S, & Idbaih A (2009) Diagnostic and prognostic markers in gliomas. *Current opinion in oncology* 21(6):537-542.

33. Sanson M*, et al.* (2009) Isocitrate dehydrogenase 1 codon 132 mutation is an important prognostic biomarker in gliomas. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 27(25):4150-4154.

34. Stupp R, Tonn JC, Brada M, Pentheroudakis G, & Group EGW (2010) High-grade malignant glioma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 21 Suppl 5:v190-193.

35. Templeton A*, et al.* (2008) Extraneural spread of glioblastoma--report of two cases. *Onkologie* 31(4):192-194.

36. Ria F*, et al.* (2001) The level of manganese superoxide dismutase content is an independent prognostic factor for glioblastoma. Biological mechanisms and clinical implications. *British journal of cancer* 84(4):529-534.

37. Ohgaki H & Kleihues P (2013) The definition of primary and secondary glioblastoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* 19(4):764-772.

38. Smeitink J (2010) Metabolism, gliomas, and IDH1. *The New England journal of medicine* 362(12):1144-1145.

39. Yan H*, et al.* (2009) IDH1 and IDH2 mutations in gliomas. *The New England journal of medicine* 360(8):765-773.

40. Kleihues P & Ohgaki H (1999) Primary and secondary glioblastomas: from concept to clinical diagnosis. *Neuro-oncology* 1(1):44-51.

41. Ohgaki H*, et al.* (2004) Genetic pathways to glioblastoma: a population-based study. *Cancer research* 64(19):6892-6899.

42. Chen J, McKay RM, & Parada LF (2012) Malignant glioma: lessons from genomics, mouse models, and stem cells. *Cell* 149(1):36-47.

43. Verhaak RG*, et al.* (2010) Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer cell* 17(1):98-110.

44. Vitucci M, Hayes DN, & Miller CR (2011) Gene expression profiling of gliomas: merging genomic and histopathological classification for personalised therapy. *British journal of cancer* 104(4):545-553.

45. Reya T, Morrison SJ, Clarke MF, & Weissman IL (2001) Stem cells, cancer, and cancer stem cells. *Nature* 414(6859):105-111.

46. Brescia P, Richichi C, & Pelicci G (2012) Current strategies for identification of glioma stem cells: adequate or unsatisfactory? *Journal of oncology* 2012:376894.

47. Altaner C (2008) Glioblastoma and stem cells. *Neoplasma* 55(5):369-374.

48. Bonnet D & Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature medicine* 3(7):730-737.

49. Clarke MF & Fuller M (2006) Stem cells and cancer: two faces of eve. *Cell* 124(6):1111-1115.

50. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, & Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America* 100(7):3983-3988.

51. Galli R*, et al.* (2004) Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer research* 64(19):7011-7021.

52. Singh SK*, et al.* (2004) Identification of human brain tumour initiating cells. *Nature* 432(7015):396-401.

53. Fang D*, et al.* (2005) A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer research* 65(20):9328-9337.

54. Zhang P, Zuo H, Ozaki T, Nakagomi N, & Kakudo K (2006) Cancer stem cell hypothesis in thyroid cancer. *Pathology international* 56(9):485-489.

55. Alamgeer M, Peacock CD, Matsui W, Ganju V, & Watkins DN (2013) Cancer stem cells in lung cancer: Evidence and controversies. *Respirology* 18(5):757-764.

56. Scaffidi P & Misteli T (2011) In vitro generation of human cells with cancer stem cell properties. *Nature cell biology* 13(9):1051-1061.

57. Zhou BB*, et al.* (2009) Tumour-initiating cells: challenges and opportunities for anticancer drug discovery. *Nature reviews. Drug discovery* 8(10):806-823.

58. Jordan CT, Guzman ML, & Noble M (2006) Cancer stem cells. *The New England journal of medicine* 355(12):1253-1261.

59. Quintana E*, et al.* (2008) Efficient tumour formation by single human melanoma cells. *Nature* 456(7222):593-598.

60. Bissell MJ & Labarge MA (2005) Context, tissue plasticity, and cancer: are tumor stem cells also regulated by the microenvironment? *Cancer cell* 7(1):17-23.

61. Mantovani A (2009) Cancer: Inflaming metastasis. *Nature* 457(7225):36-37.

62. Bao S*, et al.* (2006) Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 444(7120):756-760.

63. Ignatova TN*, et al.* (2002) Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. *Glia* 39(3):193-206.

64. Vescovi AL, Galli R, & Reynolds BA (2006) Brain tumour stem cells. *Nature reviews. Cancer* 6(6):425-436.

65. Zeppernick F*, et al.* (2008) Stem cell marker CD133 affects clinical outcome in glioma patients. *Clinical cancer research : an official journal of the American Association for Cancer Research* 14(1):123-129.

66. Bleau AM*, et al.* (2009) PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells. *Cell stem cell* 4(3):226-235.

67. Wan F, *et al.* (2010) The utility and limitations of neurosphere assay, CD133 immunophenotyping and side population assay in glioma stem cell research. *Brain pathology* 20(5):877-889.

68. Wang J, *et al.* (2008) CD133 negative glioma cells form tumors in nude rats and give rise to CD133 positive cells. *International journal of cancer. Journal international du cancer* 122(4):761-768.

69. Lathia JD, *et al.* (2010) Integrin alpha 6 regulates glioblastoma stem cells. *Cell stem cell* 6(5):421-432.

70. Read TA, *et al.* (2009) Identification of CD15 as a marker for tumor-propagating cells in a mouse model of medulloblastoma. *Cancer cell* 15(2):135-147.

71. Son MJ, Woolard K, Nam DH, Lee J, & Fine HA (2009) SSEA-1 is an enrichment marker for tumor-initiating cells in human glioblastoma. *Cell stem cell* 4(5):440-452.

72. Uchida N, *et al.* (2000) Direct isolation of human central nervous system stem cells. *Proceedings of the National Academy of Sciences of the United States of America* 97(26):14720-14725.

73. Hemmati HD, *et al.* (2003) Cancerous stem cells can arise from pediatric brain tumors. *Proceedings of the National Academy of Sciences of the United States of America* 100(25):15178-15183.

74. Laks DR, *et al.* (2009) Neurosphere formation is an independent predictor of clinical outcome in malignant glioma. *Stem cells* 27(4):980-987.

75. Pollard SM, *et al.* (2009) Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. *Cell stem cell* 4(6):568-580.

76. Sanai N, Alvarez-Buylla A, & Berger MS (2005) Neural stem cells and the origin of gliomas. *The New England journal of medicine* 353(8):811-822.

77. Weissman T, Noctor SC, Clinton BK, Honig LS, & Kriegstein AR (2003) Neurogenic radial glial cells in reptile, rodent and human: from mitosis to migration. *Cerebral cortex* 13(6):550-559.

78. Shimizu F, Watanabe TK, Shinomiya H, Nakamura Y, & Fujiwara T (1997) Isolation and expression of a cDNA for human brain fatty acid-binding protein (B-FABP). *Biochimica et biophysica acta* 1354(1):24-28.

79. Oeemig JS, *et al.* (2009) Backbone and sidechain 1H, 13C and 15N resonance assignments of the human brain-type fatty acid binding protein (FABP7) in its apo form and the holo forms binding to DHA, oleic acid, linoleic acid and elaidic acid. *Biomolecular NMR assignments* 3(1):89-93.

80. Gregg C & Weiss S (2003) Generation of functional radial glial cells by embryonic and adult forebrain neural stem cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 23(37):11587-11601.

81. Liang Y, Bollen AW, Aldape KD, & Gupta N (2006) Nuclear FABP7 immunoreactivity is preferentially expressed in infiltrative glioma and is associated with poor prognosis in EGFR-overexpressing glioblastoma. *BMC cancer* 6:97.

82. Feng L, Hatten ME, & Heintz N (1994) Brain lipid-binding protein (BLBP): a novel signaling system in the developing mammalian CNS. *Neuron* 12(4):895-908.

83. Godbout R, Bisgrove DA, Shkolny D, & Day RS, 3rd (1998) Correlation of B-FABP and GFAP expression in malignant glioma. *Oncogene* 16(15):1955-1962.

84. Miller SJ*, et al.* (2003) Brain lipid binding protein in axon-Schwann cell interactions and peripheral nerve tumorigenesis. *Molecular and cellular biology* 23(6):2213-2224.

85. Taylor MD*, et al.* (2005) Radial glia cells are candidate stem cells of ependymoma. *Cancer cell* 8(4):323-335.

86. Junttila MR & de Sauvage FJ (2013) Influence of tumour micro-environment heterogeneity on therapeutic response. *Nature* 501(7467):346-354.

87. Whiteside TL (2008) The tumor microenvironment and its role in promoting tumor growth. *Oncogene* 27(45):5904-5912.

88. Pietras K & Ostman A (2010) Hallmarks of cancer: interactions with the tumor stroma. *Experimental cell research* 316(8):1324-1331.

89. Polyak K & Kalluri R (2010) The role of the microenvironment in mammary gland development and cancer. *Cold Spring Harbor perspectives in biology* 2(11):a003244.

90. Rasanen K & Vaheri A (2010) Activation of fibroblasts in cancer stroma. *Experimental cell research* 316(17):2713-2722.

91. Spaeth EL*, et al.* (2009) Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression. *PloS one* 4(4):e4992.

92. Wels J, Kaplan RN, Rafii S, & Lyden D (2008) Migratory neighbors and distant invaders: tumor-associated niche cells. *Genes & development* 22(5):559-574.

93. Udagawa T, Puder M, Wood M, Schaefer BC, & D'Amato RJ (2006) Analysis of tumor-associated stromal cells using SCID GFP transgenic mice: contribution of local and bone marrow-derived host cells. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 20(1):95-102.

94. Kalluri R & Zeisberg M (2006) Fibroblasts in cancer. *Nature reviews. Cancer* 6(5):392-401.

95. Paunescu V*, et al.* (2011) Tumour-associated fibroblasts and mesenchymal stem cells: more similarities than differences. *Journal of cellular and molecular medicine* 15(3):635-646.

96. Dvorak HF (1986) Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *The New England journal of medicine* 315(26):1650-1659.

97. Haddow A (1972) Molecular repair, wound healing, and carcinogenesis: tumor production a possible overhealing? *Advances in cancer research* 16:181-234.

98. Mueller MM & Fusenig NE (2004) Friends or foes - bipolar effects of the tumour stroma in cancer. *Nature reviews. Cancer* 4(11):839-849.

99. Bhowmick NA*, et al.* (2004) TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* 303(5659):848-851.

100. Kabbinavar FF*, et al.* (2005) Combined analysis of efficacy: the addition of bevacizumab to fluorouracil/leucovorin improves survival for patients with metastatic colorectal cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 23(16):3706-3712.

101. Lorger M (2012) Tumor microenvironment in the brain. *Cancers* 4(1):218-243.

102. Charles NA, Holland EC, Gilbertson R, Glass R, & Kettenmann H (2011) The brain tumor microenvironment. *Glia* 59(8):1169-1180.

103. Graeber MB, Scheithauer BW, & Kreutzberg GW (2002) Microglia in brain tumors. *Glia* 40(2):252-259.

104. Watters JJ, Schartner JM, & Badie B (2005) Microglia function in brain tumors. *Journal of neuroscience research* 81(3):447-455.

105. Hanisch UK & Kettenmann H (2007) Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nature neuroscience* 10(11):1387-1394.

106. Badie B & Schartner JM (2000) Flow cytometric characterization of tumor-associated macrophages in experimental gliomas. *Neurosurgery* 46(4):957-961; discussion 961-952.

107. Alterman RL & Stanley ER (1994) Colony stimulating factor-1 expression in human glioma. *Molecular and chemical neuropathology / sponsored by the International Society for Neurochemistry and the World Federation of Neurology and research groups on neurochemistry and cerebrospinal fluid* 21(2-3):177-188.

108. Okada M*, et al.* (2009) Tumor-associated macrophage/microglia infiltration in human gliomas is correlated with MCP-3, but not MCP-1. *International journal of oncology* 34(6):1621-1627.

109. Suzuki Y, Funakoshi H, Machide M, Matsumoto K, & Nakamura T (2008) Regulation of cell migration and cytokine production by HGF-like protein (HLP) / macrophage stimulating protein (MSP) in primary microglia. *Biomedical research* 29(2):77-84.

110. Suzumura A, Sawada M, Yamamoto H, & Marunouchi T (1993) Transforming growth factor-beta suppresses activation and proliferation of microglia in vitro. *Journal of immunology* 151(4):2150-2158.

111. Badie B, Bartley B, & Schartner J (2002) Differential expression of MHC class II and B7 costimulatory molecules by microglia in rodent gliomas. *Journal of neuroimmunology* 133(1-2):39-45.

112. Bettinger I, Thanos S, & Paulus W (2002) Microglia promote glioma migration. *Acta neuropathologica* 103(4):351-355.

113. Markovic DS, Glass R, Synowitz M, Rooijen N, & Kettenmann H (2005) Microglia stimulate the invasiveness of glioma cells by increasing the activity of metalloprotease-2. *Journal of neuropathology and experimental neurology* 64(9):754-762.

114. Wen PY & Kesari S (2008) Malignant gliomas in adults. *The New England journal of medicine* 359(5):492-507.

115. Wesseling P*, et al.* (1995) Early and extensive contribution of pericytes/vascular smooth muscle cells to microvascular proliferation in glioblastoma multiforme: an immuno-light

and immuno-electron microscopic study. *Journal of neuropathology and experimental neurology* 54(3):304-310.

116. Calabrese C*, et al.* (2007) A perivascular niche for brain tumor stem cells. *Cancer cell* 11(1):69-82.

117. Folkins C*, et al.* (2007) Anticancer therapies combining antiangiogenic and tumor cell cytotoxic effects reduce the tumor stem-like cell fraction in glioma xenograft tumors. *Cancer research* 67(8):3560-3564.

118. Cleaver O & Melton DA (2003) Endothelial signaling during development. *Nature medicine* 9(6):661-668.

119. Song S, Ewald AJ, Stallcup W, Werb Z, & Bergers G (2005) PDGFRbeta+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. *Nature cell biology* 7(9):870-879.

120. Gallagher PG*, et al.* (2005) Gene expression profiling reveals cross-talk between melanoma and fibroblasts: implications for host-tumor interactions in metastasis. *Cancer research* 65(10):4134-4146.

121. Hwang RF*, et al.* (2008) Cancer-associated stromal fibroblasts promote pancreatic tumor progression. *Cancer research* 68(3):918-926.

122. Sameshima T*, et al.* (2000) Glioma cell extracellular matrix metalloproteinase inducer (EMMPRIN) (CD147) stimulates production of membrane-type matrix metalloproteinases and activated gelatinase A in co-cultures with brain-derived fibroblasts. *Cancer letters* 157(2):177-184.

123. Bourkoula E*, et al.* (2014) Glioma-associated stem cells: a novel class of tumor-supporting cells able to predict prognosis of human low-grade gliomas. *Stem cells* 32(5):1239-1253.

124. Sofroniew MV & Vinters HV (2010) Astrocytes: biology and pathology. *Acta neuropathologica* 119(1):7-35.

125. Barker AJ & Ullian EM (2008) New roles for astrocytes in developing synaptic circuits. *Communicative & integrative biology* 1(2):207-211.

126. Laird MD, Vender JR, & Dhandapani KM (2008) Opposing roles for reactive astrocytes following traumatic brain injury. *Neuro-Signals* 16(2-3):154-164.

127. Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, & Alvarez-Buylla A (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97(6):703-716.

128. Lim DA, Huang YC, & Alvarez-Buylla A (2007) The adult neural stem cell niche: lessons for future neural cell replacement strategies. *Neurosurgery clinics of North America* 18(1):81-92, ix.

129. Robel S, Berninger B, & Gotz M (2011) The stem cell potential of glia: lessons from reactive gliosis. *Nature reviews. Neuroscience* 12(2):88-104.

130. Garcia AD, Doan NB, Imura T, Bush TG, & Sofroniew MV (2004) GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. *Nature neuroscience* 7(11):1233-1241.

131. Sofroniew MV (2009) Molecular dissection of reactive astrogliosis and glial scar formation. *Trends in neurosciences* 32(12):638-647.

132. Lang B*, et al.* (2004) Astrocytes in injured adult rat spinal cord may acquire the potential of neural stem cells. *Neuroscience* 128(4):775-783.

133. Buffo A*, et al.* (2008) Origin and progeny of reactive gliosis: A source of multipotent cells in the injured brain. *Proceedings of the National Academy of Sciences of the United States of America* 105(9):3581-3586.

134. Hoelzinger DB, Demuth T, & Berens ME (2007) Autocrine factors that sustain glioma invasion and paracrine biology in the brain microenvironment. *Journal of the National Cancer Institute* 99(21):1583-1593.

135. Lee J*, et al.* (2009) Glioma-induced remodeling of the neurovascular unit. *Brain research* 1288:125-134.

136. Le DM*, et al.* (2003) Exploitation of astrocytes by glioma cells to facilitate invasiveness: a mechanism involving matrix metalloproteinase-2 and the urokinase-type plasminogen activator-plasmin cascade. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 23(10):4034-4043.

137. Clement V, Sanchez P, de Tribolet N, Radovanovic I, & Ruiz i Altaba A (2007) HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity. *Current biology : CB* 17(2):165-172.

138. Dragovic RA*, et al.* (2011) Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. *Nanomedicine : nanotechnology, biology, and medicine* 7(6):780-788.

139. Kalra H*, et al.* (2012) Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. *PLoS biology* 10(12):e1001450.

140. Johnstone RM, Adam M, Hammond JR, Orr L, & Turbide C (1987) Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *The Journal of biological chemistry* 262(19):9412-9420.

141. Mathivanan S & Simpson RJ (2009) ExoCarta: A compendium of exosomal proteins and RNA. *Proteomics* 9(21):4997-5000.

142. Simons M & Raposo G (2009) Exosomes--vesicular carriers for intercellular communication. *Current opinion in cell biology* 21(4):575-581.

143. Futter CE, Pearse A, Hewlett LJ, & Hopkins CR (1996) Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes. *The Journal of cell biology* 132(6):1011-1023.

144. Mullock BM, Bright NA, Fearon CW, Gray SR, & Luzio JP (1998) Fusion of lysosomes with late endosomes produces a hybrid organelle of intermediate density and is NSF dependent. *The Journal of cell biology* 140(3):591-601.

145. Kowal J, Tkach M, & Thery C (2014) Biogenesis and secretion of exosomes. *Current opinion in cell biology* 29:116-125.

146. Katzmann DJ, Odorizzi G, & Emr SD (2002) Receptor downregulation and multivesicular-body sorting. *Nature reviews. Molecular cell biology* 3(12):893-905.

147. Thery C*, et al.* (2001) Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *Journal of immunology* 166(12):7309-7318.

148. van Blitterswijk WJ, van der Luit AH, Veldman RJ, Verheij M, & Borst J (2003) Ceramide: second messenger or modulator of membrane structure and dynamics? *The Biochemical journal* 369(Pt 2):199-211.

149. Trajkovic K*, et al.* (2008) Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* 319(5867):1244-1247.

150. van Niel G*, et al.* (2011) The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis. *Developmental cell* 21(4):708-721.

151. Rodriguez-Boulan E, Kreitzer G, & Musch A (2005) Organization of vesicular trafficking in epithelia. *Nature reviews. Molecular cell biology* 6(3):233-247.

152. Hutagalung AH & Novick PJ (2011) Role of Rab GTPases in membrane traffic and cell physiology. *Physiological reviews* 91(1):119-149.

153. Savina A, Vidal M, & Colombo MI (2002) The exosome pathway in K562 cells is regulated by Rab11. *Journal of cell science* 115(Pt 12):2505-2515.

154. Ostrowski M*, et al.* (2010) Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nature cell biology* 12(1):19-30; sup pp 11-13.

155. King HW, Michael MZ, & Gleadle JM (2012) Hypoxic enhancement of exosome release by breast cancer cells. *BMC cancer* 12:421.

156. Mittelbrunn M*, et al.* (2011) Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nature communications* 2:282.

157. Escrevente C, Keller S, Altevogt P, & Costa J (2011) Interaction and uptake of exosomes by ovarian cancer cells. *BMC cancer* 11:108.

158. Feng D*, et al.* (2010) Cellular internalization of exosomes occurs through phagocytosis. *Traffic* 11(5):675-687.

159. Simpson RJ, Lim JW, Moritz RL, & Mathivanan S (2009) Exosomes: proteomic insights and diagnostic potential. *Expert review of proteomics* 6(3):267-283.

160. Parolini I*, et al.* (2009) Microenvironmental pH is a key factor for exosome traffic in tumor cells. *The Journal of biological chemistry* 284(49):34211-34222.

161. Chevallier J*, et al.* (2008) Lysobisphosphatidic acid controls endosomal cholesterol levels. *The Journal of biological chemistry* 283(41):27871-27880.

162. Valadi H*, et al.* (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature cell biology* 9(6):654-659.

163. Skog J*, et al.* (2008) Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nature cell biology* 10(12):1470-1476.

164. Guescini M, Genedani S, Stocchi V, & Agnati LF (2010) Astrocytes and Glioblastoma cells release exosomes carrying mtDNA. *Journal of neural transmission* 117(1):1-4.

165. Guescini M*, et al.* (2010) C2C12 myoblasts release micro-vesicles containing mtDNA and proteins involved in signal transduction. *Experimental cell research* 316(12):1977-1984.

166. Kahlert C & Kalluri R (2013) Exosomes in tumor microenvironment influence cancer progression and metastasis. *Journal of molecular medicine* 91(4):431-437.

167. Azmi AS, Bao B, & Sarkar FH (2013) Exosomes in cancer development, metastasis, and drug resistance: a comprehensive review. *Cancer metastasis reviews* 32(3-4):623-642.

168. Al-Nedawi K*, et al.* (2008) Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nature cell biology* 10(5):619-624.

169. Demory Beckler M*, et al.* (2013) Proteomic analysis of exosomes from mutant KRAS colon cancer cells identifies intercellular transfer of mutant KRAS. *Molecular & cellular proteomics : MCP* 12(2):343-355.

170. Webber J, Steadman R, Mason MD, Tabi Z, & Clayton A (2010) Cancer exosomes trigger fibroblast to myofibroblast differentiation. *Cancer research* 70(23):9621-9630.

171. Vong S & Kalluri R (2011) The role of stromal myofibroblast and extracellular matrix in tumor angiogenesis. *Genes & cancer* 2(12):1139-1145.

172. Al-Nedawi K, Meehan B, Kerbel RS, Allison AC, & Rak J (2009) Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. *Proceedings of the National Academy of Sciences of the United States of America* 106(10):3794-3799.

173. Zhuang G*, et al.* (2012) Tumour-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway. *The EMBO journal* 31(17):3513-3523.

174. Hakulinen J, Sankkila L, Sugiyama N, Lehti K, & Keski-Oja J (2008) Secretion of active membrane type 1 matrix metalloproteinase (MMP-14) into extracellular space in microvesicular exosomes. *Journal of cellular biochemistry* 105(5):1211-1218.

175. McCready J, Sims JD, Chan D, & Jay DG (2010) Secretion of extracellular hsp90alpha via exosomes increases cancer cell motility: a role for plasminogen activation. *BMC cancer* 10:294.

176. Bobrie A*, et al.* (2012) Rab27a supports exosome-dependent and -independent mechanisms that modify the tumor microenvironment and can promote tumor progression. *Cancer research* 72(19):4920-4930.

177. Fabbri M*, et al.* (2012) MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. *Proceedings of the National Academy of Sciences of the United States of America* 109(31):E2110-2116.

178. Chalmin F*, et al.* (2010) Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. *The Journal of clinical investigation* 120(2):457-471.

179. Nagaraj S & Gabrilovich DI (2012) Regulation of suppressive function of myeloid-derived suppressor cells by CD4+ T cells. *Seminars in cancer biology* 22(4):282-288.

180. Kim HK, *et al.* (2003) Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in patients with gastric cancer: possible role of a metastasis predictor. *European journal of cancer* 39(2):184-191.

181. Mellinghoff IK, *et al.* (2005) Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *The New England journal of medicine* 353(19):2012-2024.

182. Silva J, *et al.* (2012) Analysis of exosome release and its prognostic value in human colorectal cancer. *Genes, chromosomes & cancer* 51(4):409-418.

183. Taylor DD & Gercel-Taylor C (2008) MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecologic oncology* 110(1):13-21.

184. Peinado H, *et al.* (2012) Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nature medicine* 18(6):883-891.

185. Gallo A, Tandon M, Alevizos I, & Illei GG (2012) The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. *PloS one* 7(3):e30679.

186. Keller S, Ridinger J, Rupp AK, Janssen JW, & Altevogt P (2011) Body fluid derived exosomes as a novel template for clinical diagnostics. *Journal of translational medicine* 9:86.

187. McCarthy BJ, Kruchko C, & Central Brain Tumor Registry of the United S (2005) Consensus conference on cancer registration of brain and central nervous system tumors. *Neuro-oncology* 7(2):196-201.

188. Hegi ME, *et al.* (2005) MGMT gene silencing and benefit from temozolomide in glioblastoma. *The New England journal of medicine* 352(10):997-1003.

189. Demuth T & Berens ME (2004) Molecular mechanisms of glioma cell migration and invasion. *Journal of neuro-oncology* 70(2):217-228.

190. Molina JR, Hayashi Y, Stephens C, & Georgescu MM (2010) Invasive glioblastoma cells acquire stemness and increased Akt activation. *Neoplasia* 12(6):453-463.

191. Scherer HJ (1940) A Critical Review: The Pathology of Cerebral Gliomas. *Journal of neurology and psychiatry* 3(2):147-177.

192. Pollard JW (2004) Tumour-educated macrophages promote tumour progression and metastasis. *Nature reviews. Cancer* 4(1):71-78.

193. Cesselli D, *et al.* (2011) Role of tumor associated fibroblasts in human liver regeneration, cirrhosis, and cancer. *International journal of hepatology* 2011:120925.

194. Franco OE, Shaw AK, Strand DW, & Hayward SW (2010) Cancer associated fibroblasts in cancer pathogenesis. *Seminars in cell & developmental biology* 21(1):33-39.

195. Rana S, Malinowska K, & Zoller M (2013) Exosomal tumor microRNA modulates premetastatic organ cells. *Neoplasia* 15(3):281-295.

196. Boelens MC, *et al.* (2014) Exosome transfer from stromal to breast cancer cells regulates therapy resistance pathways. *Cell* 159(3):499-513.

197. Liang CC, Park AY, & Guan JL (2007) In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature protocols* 2(2):329-333.

198. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4):402-408.

199. Galli R (2013) The neurosphere assay applied to neural stem cells and cancer stem cells. *Methods in molecular biology* 986:267-277.

200. Beltrami AP*, et al.* (2007) Multipotent cells can be generated in vitro from several adult human organs (heart, liver, and bone marrow). *Blood* 110(9):3438-3446.

201. Raposo G & Stoorvogel W (2013) Extracellular vesicles: exosomes, microvesicles, and friends. *The Journal of cell biology* 200(4):373-383.

202. San Francisco IF, DeWolf WC, Peehl DM, & Olumi AF (2004) Expression of transforming growth factor-beta 1 and growth in soft agar differentiate prostate carcinoma-associated fibroblasts from normal prostate fibroblasts. *International journal of cancer. Journal international du cancer* 112(2):213-218.

203. Chen F, Qiu J, Wang J, Liu S, & Mi W (2006) [Isolation and culture of neural stem cell from rat olfactory epithelium]. *Lin chuang er bi yan hou ke za zhi = Journal of clinical otorhinolaryngology* 20(14):656-659.

204. Nat R*, et al.* (2007) Neurogenic neuroepithelial and radial glial cells generated from six human embryonic stem cell lines in serum-free suspension and adherent cultures. *Glia* 55(4):385-399.

205. Marko K*, et al.* (2011) Isolation of radial glia-like neural stem cells from fetal and adult mouse forebrain via selective adhesion to a novel adhesive peptide-conjugate. *PloS one* 6(12):e28538.

206. Englund C*, et al.* (2005) Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 25(1):247-251.

207. Noctor SC, Flint AC, Weissman TA, Dammerman RS, & Kriegstein AR (2001) Neurons derived from radial glial cells establish radial units in neocortex. *Nature* 409(6821):714-720.

208. Heins N*, et al.* (2002) Glial cells generate neurons: the role of the transcription factor Pax6. *Nature neuroscience* 5(4):308-315.

209. Sofroniew MV (2005) Reactive astrocytes in neural repair and protection. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* 11(5):400-407.

210. Fontana A, Fierz W, & Wekerle H (1984) Astrocytes present myelin basic protein to encephalitogenic T-cell lines. *Nature* 307(5948):273-276.

211. Yong VW, Yong FP, Ruijs TC, Antel JP, & Kim SU (1991) Expression and modulation of HLA-DR on cultured human adult astrocytes. *Journal of neuropathology and experimental neurology* 50(1):16-28.

212. Wilhelmsson U*, et al.* (2006) Redefining the concept of reactive astrocytes as cells that remain within their unique domains upon reaction to injury. *Proceedings of the National Academy of Sciences of the United States of America* 103(46):17513-17518.

213. Nicolson GL, *et al.* (1996) Brain metastasis: role of trophic, autocrine, and paracrine factors in tumor invasion and colonization of the central nervous system. *Current topics in microbiology and immunology* 213 (Pt 2):89-115.

214. Lorger M & Felding-Habermann B (2010) Capturing changes in the brain microenvironment during initial steps of breast cancer brain metastasis. *The American journal of pathology* 176(6):2958-2971.

215. Sierra A, *et al.* (1997) Astrocyte-derived cytokines contribute to the metastatic brain specificity of breast cancer cells. *Laboratory investigation; a journal of technical methods and pathology* 77(4):357-368.

216. Langley RR, *et al.* (2009) Generation of an immortalized astrocyte cell line from H-2Kb-tsA58 mice to study the role of astrocytes in brain metastasis. *International journal of oncology* 35(4):665-672.

217. Marchetti D, Li J, & Shen R (2000) Astrocytes contribute to the brain-metastatic specificity of melanoma cells by producing heparanase. *Cancer research* 60(17):4767-4770.

218. Bechmann I, *et al.* (2002) Astrocyte-induced T cell elimination is CD95 ligand dependent. *Journal of neuroimmunology* 132(1-2):60-65.

219. D'Souza-Schorey C & Clancy JW (2012) Tumor-derived microvesicles: shedding light on novel microenvironment modulators and prospective cancer biomarkers. *Genes & development* 26(12):1287-1299.

220. Svensson KJ, *et al.* (2011) Hypoxia triggers a proangiogenic pathway involving cancer cell microvesicles and PAR-2-mediated heparin-binding EGF signaling in endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* 108(32):13147-13152.

221. Bastida E, Ordinas A, Escolar G, & Jamieson GA (1984) Tissue factor in microvesicles shed from U87MG human glioblastoma cells induces coagulation, platelet aggregation, and thrombogenesis. *Blood* 64(1):177-184.

222. Morello M, *et al.* (2013) Large oncosomes mediate intercellular transfer of functional microRNA. *Cell cycle* 12(22):3526-3536.

223. Ridet JL, Malhotra SK, Privat A, & Gage FH (1997) Reactive astrocytes: cellular and molecular cues to biological function. *Trends in neurosciences* 20(12):570-577.

224. Buffo A, *et al.* (2005) Expression pattern of the transcription factor Olig2 in response to brain injuries: implications for neuronal repair. *Proceedings of the National Academy of Sciences of the United States of America* 102(50):18183-18188.

225. Katz AM, *et al.* (2012) Astrocyte-specific expression patterns associated with the PDGF-induced glioma microenvironment. *PloS one* 7(2):e32453.

226. Park D, *et al.* (2010) Nestin is required for the proper self-renewal of neural stem cells. *Stem cells* 28(12):2162-2171.

227. Elkabetz Y, *et al.* (2008) Human ES cell-derived neural rosettes reveal a functionally distinct early neural stem cell stage. *Genes & development* 22(2):152-165.

228. Seri B, Garcia-Verdugo JM, McEwen BS, & Alvarez-Buylla A (2001) Astrocytes give rise to new neurons in the adult mammalian hippocampus. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 21(18):7153-7160.

229. Ganat YM*, et al.* (2006) Early postnatal astroglial cells produce multilineage precursors and neural stem cells in vivo. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 26(33):8609-8621.

230. Yang C*, et al.* (2012) beta-Catenin signaling initiates the activation of astrocytes and its dysregulation contributes to the pathogenesis of astrocytomas. *Proceedings of the National Academy of Sciences of the United States of America* 109(18):6963-6968.

231. Kurtz A*, et al.* (1994) The expression pattern of a novel gene encoding brain-fatty acid binding protein correlates with neuronal and glial cell development. *Development* 120(9):2637-2649.

232. Schmechel DE & Rakic P (1979) A Golgi study of radial glial cells in developing monkey telencephalon: morphogenesis and transformation into astrocytes. *Anatomy and embryology* 156(2):115-152.

233. Goldman S (2003) Glia as neural progenitor cells. *Trends in neurosciences* 26(11):590-596.

234. Mita R*, et al.* (2007) B-FABP-expressing radial glial cells: the malignant glioma cell of origin? *Neoplasia* 9(9):734-744.

PUBLICATIONS

a. Publications regarding the PhD Thesis

Bourkoula E, Mangoni D, Ius T, Pucer A, Isola M, Musiello D, Marzinotto S, Toffoletto B, Sorrentino M, Palma A, Caponnetto F, Gregoraci G, Vindigni M, Pizzolitto S, Falconieri G, De Maglio G, Pecile V, Ruaro ME, Gri G, Parisse P, Casalis L, Scoles G, Skrap M, Beltrami CA, Beltrami AP, Cesselli D. Glioma-associated stem cells: a novel class of tumor-supporting cells able to predict prognosis of human low-grade gliomas. Stem Cells. 2014 May;32(5):1239-53. doi: 10.1002/stem.1605.

b. Other publications

Domenis R, Lazzaro L, Calabrese S, Mangoni D, Gallelli A, Bourkoula E, Manini I, Bergamin N, Toffoletto B, Beltrami CA, Beltrami AP, Cesselli D, Parodi PC. Adipose tissue derived stem cells: in vitro and in vivo analysis of a standard- and three commercially available Cell-Assisted Lipotransfer techniques. Stem Cell Research and Therapy. 2014 December, Stem Cell Research and Therapy, *accepted*.