DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

MiRNAs expression profiling and modulation in Glioblastoma Stem Cells

Avaliação do perfil de expressão de miRNAs e sua modulação em Células Estaminais de Glioblastoma

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Doutora Ana Luísa (Centro de Neurociências e Biologia Celular), Doutora Maria Conceição Pedroso Lima (Centro de Neurociências e Biologia Celular) e Maria Amália da Silva Jurado (Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra)

Rúben Miguel Gonçalves Branco

2014

This Work was performed at the Center for Neuroscience and Cell Biology, University of Coimbra, Portugal in the group of Vectors and Gene Therapy.

UNIVERSIDADE DE COIMBRA

A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original.

Albert Einstein

Agradecimentos

Apesar do processo solitário a que qualquer investigador está destinado, durante a minha dissertação de mestrado reuni contributos de várias pessoas. Desta forma, deixo apenas algumas palavras a essas pessoas que, ao longo do meu percurso no Mestrado de Bioquímica pela Universidade de Coimbra, directa ou indirectamente, me ajudaram a cumprir os objectivos e a realizar mais esta etapa da minha formação académica. Sem os vossos contributos, esta dissertação não teria sido possível.

Começaria por agradecer à Doutora Maria da Conceição Pedroso de Lima por me ter dado a oportunidade de trabalhar naquilo que mais gosto. Agradeço também toda a preocupação, seu rigor e conhecimento ciêntifico. Para mim é um orgulho ter sido seu aluno. Para si, o meu mais sincero obrigado.

Não poderia deixar de agradecer à Ana Luisa por toda a ajuda e incrível disponibilidade, em todas as fases desta tese. Por todo seu apoio e por me dar todas as condições para trabalhar de forma eficiente durante a minha estadia no laboratório. Quero deixar um agradecimento especial ao Pedro Costa por toda a orientação em todos os passos desta tese. Grande parte do meu crescimento como cientista durante este ano deve-se a ti. Quero também agradecer aos meus restantes colegas do grupo de Vectores e Terapia Génica e do Centro de Neurociências e Biologia Celular. À Joana Guedes por me ter ajudado a dar os primeiros passos no laboratório. À Catarina Morais por todas as perguntas pertinentes. À Ana Teresa por todos os momentos de boa disposição. Agradeço também a todo o pessoal da "salinha dos fixes" por nunca me deixarem trabalhar sem uma gargalhada.

Aos meus colegas do curso de Bioquímica, em especial ao Rui Silva, Carlos Paula, JP, José Dias e Nuno Apóstolo por me terem aturado diariamente ao longo destes 5 anos. Aos restantes amigos de facultade, Pombo, Dani, Bruno e Helena. A todos vós agradeço por a amizade e por todos os momentos partilhados ao longo destes últimos anos.

A todos os meus colegas do Grupo Desportivo de Alcaravela, em especial ao Rafa, Cláudio e André, por todos os fim de semana de descontracção que me proporcionaram, mesmo quando os resultados não eram os melhores.

Um agradecimento a toda a minha família, em especial à minha Mãe, por todos os sacrificios, bem como toda a confiança que sempre demonstrou em mim. A pessoa que sou hoje é o reflexo da educação que me deste.

Esta tese é vos dedicada!

Table of Contents

Abbreviations

ABC	Adenosine triphosphate-binding cassette
BBB	Blood brain barrier
BMI-1	B lymphoma Mo-MLV insertion region 1 homolog
CNS	Central nervous system
CSCs	Cancer stem cells
EGFR	Epidermal growth factor receptor
FBS	Fetal Bovine Serum
GBM	Glioblastoma multiforme
GSCs	Glioma stem cells
MACS	Magnetic associated cell sorting
MHC	Major histocompatibility complex
MMP2	Matrix metalloproteinase 2
PBS	Phosphate-buffered saline
PCR	Polimerase Chain Reaction
PDGF-R	Platelet-derived growth factor receptor
PTEN	Phosphatase and tensin homolog
qRT PCR	Quantitative real time-polymerase chain reaction
RB	Retinoblastoma protein
RTKs	Tyrosine kinases receptors
STAT3	Signal transducer and activator of transcription 3
STK	Specific protein kinase
TMZ	Temozolodime
TP53	Tumor protein 53

VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization

Abstract

Among all brain cancers, glioblastoma multiforme (GBM) is the most common, malignant and lethal type of tumor. Standard treatment consists on the removal of the tumor mass with surgery, followed by chemotherapy and radiotherapy. Despite the recent advances in therapy, the life expectancy of GBM patients after diagnosis is very low. For this reason, new therapeutic approaches for GBM are urgently needed.

The discovery of cancer stem cells opens the possibility for new types of therapy. Beyond their capacity for self-renewal and tumorigenesis, these cells are known for their high resistance to radiotherapy and chemotherapy, when compared to other cancer cells. Since these cells can remain in the tissue and form a new tumor even after treatment, it seems essential to develop therapeutic strategies that target cancer stem cells, with the ultimate goal of eradicating the tumor. In this regard, miRNAs have received special attention from the scientific community in recent years. A large number of studies has suggested that miRNAs play important roles in the development of malignant gliomas. Taking this into account, therapies for GBM based on miRNA modulation are a promising field of research.

In this study, we proposed to isolate and characterize the glioblastoma stem cell (GSCs) population present in the U87 human glioblastoma cell line. Our results showed that cells isolated from this cell line, using magnetic CD133-microbeads, express nestin and CD133, two well established cancer stem cells markers, and grow in the form of neurospheres in low-adhesion conditions. Our second goal was to compare the miRNA profile of GCSs and other GBM cells and assess the potential of miRNA modulation in the GSCs, with therapeutic purposes. We found that $CD133^+$ and $CD133^-$ cells showed different miRNA profiles, especially in what concerns miR-128 expression, since this miRNA was highly downregulated in $CD133^+$ cells.

We also evaluated the effect of miR-128 overexpression, alone or in combination with the drug sunitinib, in GBM tumor cell viability. These experiments allowed us to demonstrate that miR-128 overexpression sensitized U87 cells to sunitinib-induced cell death.

Since we were unable to deliver miR-128 mimics to the GSC population using commercially available nucleic acid delivery systems, we developed preliminary studies aiming at evaluating the possibility of using stable nucleic acid delivery particles, coupled

to the chlorotoxin peptide, to perform miRNA modulation in these cells. We showed that these nanoparticles were able to deliver miRNA mimics to GSCs with high efficiency.

Overall, we found evidences that point to an important role of miRNAs in GSC stem properties and that may help to clarify the contribution of these cells to tumor progression, paving the way to the development of new miRNA-based therapeutic strategies for GBM treatment.

KEY WORDS: Glioblastoma multiforme, cancer stem cells, microRNAs, gene therapy, therapeutic resistance

Resumo

Entre todos os tipos de cancro de cérebro, o glioblastoma multiforme (GBM) é o tipo de tumor mais comum, maligno e letal. O tratamento padrão para este tipo de cancro consiste na remoção do tumor através de cirurgia, seguida de quimioterapia e radioterapia. Apesar dos avanços recentes nas formas de terapia disponíveis para esta doença, a esperança média de vida após o diagnóstico dos pacientes com GBM é muito baixo. Por esta razão, é necessário o desenvolvimento urgente de novas abordagens terapêuticas para GBM.

A descoberta da existência de células estaminais cancerígenas abriu a possibilidade para o desenvolvimento de novos tipos de terapia. Para além da sua capacidade de auto-renovação e tumorigénese, estas células são conhecidas pela sua elevada resistência à radioterapia e quimioterapia, quando comparadas com outras células cancerígenas. Uma vez que estas células podem permanecer no tecido e formar um novo tumor, mesmo após o tratamento, parece essencial o desenvolvimento de estratégias terapêuticas que visam a eliminação das células estaminais cancerigenas, com o objetivo final de erradicar o tumor. A este respeito, os miRNAs tem recebido uma atenção especial por parte da comunidade científica nos últimos anos. Um grande número de estudos tem sugerido que os miRNAs podem desempenhar papéis importantes no desenvolvimento do glioblastoma e outros gliomas. Tendo isto em conta, as terapias contra o GBM com base na modulação miRNAs são um campo promissor de pesquisa.

O objectivo principal deste trabalho consistiu no isolamento e caracterização da população de GSCs a partir da linha celular de glioblastoma humano U87. Os nossos resultados mostraram que as células isoladas desta linha cellular através do uso de microbeads magnéticas anti-CD133, expressavam nestina e CD133, dois marcadores bem estudados das GSCs, e eram capazes de crescer na forma de neuroesferas, em condições de não aderência. O nosso segundo objetivo passou por comparar o perfil de expressão de miRNAs das GCSs e de outras células de GBM, e avaliar a possibilidade de modulação de miRNAs nas GSC com um propósito terapêutico. As células $CD133^{+}$ e as células $CD133^{-}$ mostraram diferentes perfis de expressão de miRNAs, especialmente no que diz respeito à expressão do miR-128, que se encontrava significantemente reduzido nas células $CD133^{+}$.

Também foi avaliado o efeito da sobreexpressão do miR-128, sozinho ou em combinação com o fármaco sunitinib na viabilidade das células tumorais de GBM. Estas experiências

permitiram-nos demonstrar que o aumento dos níveis do miR-128, por si só ou em combinação com a droga sunitinib, sensibilizaram as células U87 para a morte celular induzida pelo sunitinib.

Devido à incapacidade de entregar os oligonucelótidos miméticos do miR-128 à população de GSCs usando sistemas de entrega de ácidos nucleicos comerciais, desenvolvemos estudos preliminares visando avaliar a possibilidade de utilização de partículas estáveis de entrega de ácidos nucléicos, acopladas ao peptideo clorotoxina, para executar a modulação dos miRNAs nestas células. Mostrámos que estas nanopartículas são capazes de entregar os oligonucelótidos miméticos do miR-128 com elevada eficiência.

Em conclusão, encontrámos evidências que apontam para um papel importante dos miRNAs nas propriedades estaminais das GSCs e que podem ajudar a esclarecer a contribuição destas células para a progressão do tumor, abrindo o caminho para o desenvolvimento de novas estratégias terapêuticas para GBM baseadas na modulação de miRNAs.

PALAVRAS-CHAVE: Glioblastoma multiforme, células estaminais cancerígenas, microRNAs, terapia génica, resistência terapêutica

Chapter 1 Introduction

1.1) Glioblastoma Multiforme

Neurons and glia are the main cell types present in the central nervous system (CNS). Neurons are able to process and transmit information through electrical and chemical signals. Glial cells (astrocytes, oligodendrocytes and microglia) are important for neuron protection as well as for the metabolic and structural support of the nervous system. The most common malignancies in the central nervous system (CNS) are gliomas, which are a group of tumors that arise from glial cells[1]. Based on their degree of malignancy and genetic alterations, gliomas can be divided in four grades according to the World Health Organization (WHO) as is shown in table 1. Grade I gliomas, also known as Pilocytic Astrocytomas and Grade II gliomas have a slow growth when compared to the other Grades. Grade III have increased anaplasia and proliferation over grades I and II and present higher mortality. Grade IV is the most malignant, showing vascular proliferation and necrosis. Glioblastoma (GBM) also known as Glioblastoma multiforme is one of the deadliest tumors and has the higher occurrence between brain tumors. Glioblastoma multiforme (GBM) remains the most malignant and frequent (20 % of intracranial tumors) of gliomas, with a life expectancy of 16 months after the diagnosis, despite current advances in therapy[1–3]. The major sites for GBM occurrence are the cerebral hemispheres and, less commonly, the brain stem, cerebellum, and spinal cord[4].

Table 1 – WHO grading system for gliomas[1,3]

Glioma Grade	Observations
Grade I (juvenile pilocytic astrocytoma)	Associated with long-term survival; benign; slow-growing tumor; less likely recurrence; low proliferative potential; Possibility of cure after surgical resection.
Grade II (astrocytoma)	Can recur as a higher grade; no necrosis; low proliferative potential
Grade III (anaplastic astrocytoma)	Mitosis occurs at a higher rate; no necrosis; high rate of recurrence; evidences of malignancy (increased mitotic activity)
Grade IV (glioblastoma)	Very high rate of mitosis; presence of vascular proliferation; necrosis; evidences of malignancy (mitotically very active)

The major hallmarks of GBMs are its high ability to spread to the nearby tissue, uncontrolled cellular proliferation, high angiogenesis, resistance to apoptosis and genetic instability[2].

1.1.2) GBM Classification

GBMs can be primary or secondary (figure 1), depending on the origin and development of the tumor. The primary or "de novo" subtype appears without prior lesions, it is more frequent and usually affects the elderly. The secondary or progressive subtype arises from lower grade astrocytomas. Phosphatase and tensin homolog (PTEN) mutations and epidermal growth factor receptor (EGFR) amplification are associated with primary GBMs. On the other hand, tumor protein 53 (TP53) mutations are involved in the pathways leading to the secondary subtype[5,6].

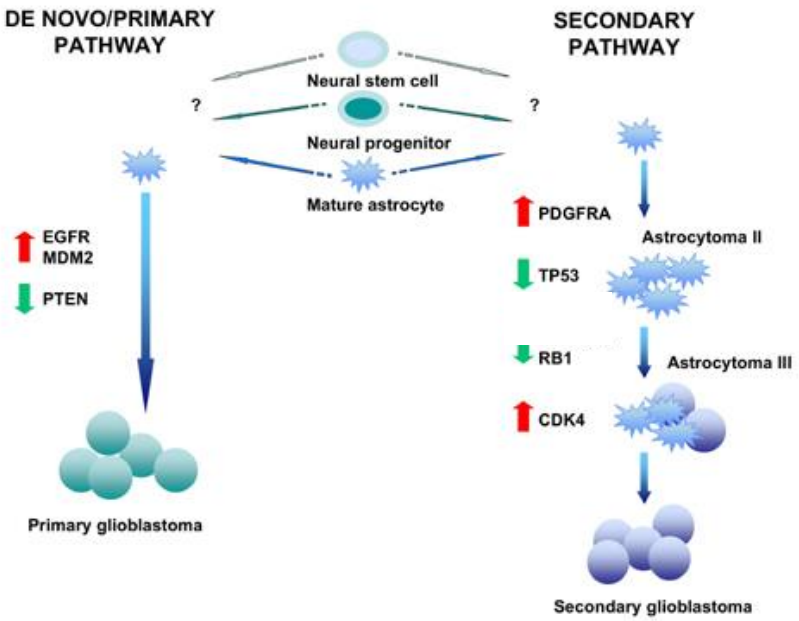


Figure 1. Molecular genetic pathways leading to glioblastoma multiforme. GBM can be classified as primary or secondary depending on the characteristics and formation of the tumor. There are several mutations usually associated with GBM formation. For primary GBM, increased expression of EGFR and MDM2 and downregulation of PTEN are often found. The secondary pathway is more complex, usually presenting increased expression of PDGF/CDK4 and low expression of TP53.

1.1.3) GBM Hallmarks

There are a large number of regulatory pathways which are essential to maintain the cellular environment, controlling the balance of cellular growth/death. In GBM, there several molecular variations can cause the impairment of this balance. There are different types of cells within the tumor, varying in morphology, genetics and biological behavior[7,8]. This heterogeneity makes this tumor particularly difficult to treat, since different cells respond in different ways to the available therapeutic aproaches. Tumor heterogeneity may arise from the accumulation of different mutations that result in genetic variability. Some researches suggests that this heterogeneity is due to a specific group of cells within the tumor, the cancer stem cells (CSCs)[9–11]. These authors also suggest that these cells are important for maintenance of the tumor self-renewal and to development of resistance to different types of treatment[12,13]. Despite recent advances in this field of research, the role of CSCs in GBM development and maintenance remains unclear.

1.1.3.1) Molecular Pathways involved in gliomas

Neoplastic transformation of gliomas progresses through several stages of intracellular events: 1) acquisition of invasive properties, 2) activation of cell proliferation signals, 3) loss of cell cycle control, 4) upregulated angiogenesis and 5) deregulation of apoptosis. These hallmarks, summarized in figure 2, are due to the highly unstable genome of GBM, which is responsible for making it the most malignant and aggressive type of brain tumor[7,14].

The invasive capacity of GBM is due to its ability to migrate to nearby tissue and modulate the extracellular space. Glioma invasion is a complex process involving detachment from the original site, adhesion and remodeling of the extracellular matrix and cell migration[15]. Proteases seem to play an important role in this process. These proteins degrade the extracellular environment, allowing the tumor to grow and also promoting cell migration. Several studies show that three specific proteases are found in high levels in gliomas: matrix metalloproteinase 2 (MMP2), the serine protease urokinase-type plasminogen activator and its receptor, and the cysteine protease cathepsin

B[7,14]. Despite being highly invasive, GBM does not metastasize to other organs[2]. Many membrane proteins contribute to invasion signaling in GBM, such as tyrosine kinases receptors (RTKs), integrin and CD44. Amplification of the epidermal growth factor receptor (EGFR) gene is the most common alteration observed in this type of tumor. This overexpression of EGFR was shown to be associated with upregulation of multiple genes

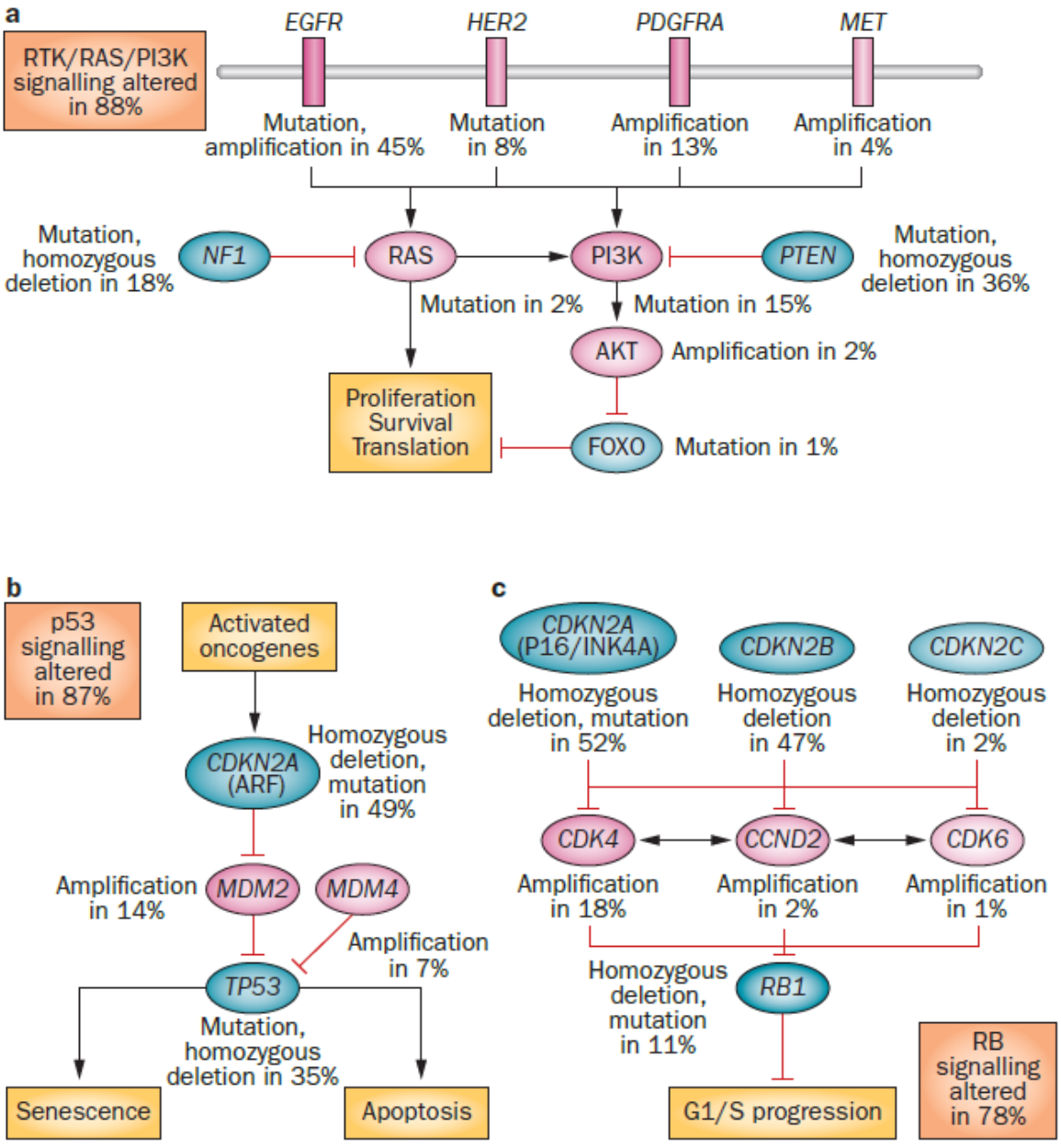


Figure 2. Signaling pathways altered in malignant gliomas. Sequence changes and copy number in three major signaling pathways associated with GBM: **a)** RTK/RAS/PI3K, **b)** p53 and **c)** Rb. Blue indicates inactivating alterations while red indicates activating alterations. The percentages of tumors affected and the nature of the alteration can be seen below. Red boxes comprise the final percentages of glioblastomas. Adapted from [121]

associated with invasion, including metaloproteases and collagens[16]. In addition, studies based on EFGR inhibition had successful results in delaying the invasion capacity of GBM[14]. Integrins are transmembrane heterodimers that link actin filaments of cytoskeleton to the extracellular matrix[17]. β1 subunits of integrin are important for the invasive capacity of gliomas. It was shown that α3β1 is over-expressed and is a key regulator of glioma cell migration[18]. In addition, CD44, a transmembrane glycoprotein, in highly expressed in all glioma types. In tumor cells, CD44 is cleaved inducing cell detachment from hyaluronic acid and promotes cell migration[19].

Strong proliferative activity is prominent is almost all GBM cases. GBM growth and progression depends of the activity of certain surface receptors that control internal signaling pathways, such as the RTKs and Serine/threonine specific protein kinase (STK)[20]. For instance, the gene PTEN, which encodes a tyrosine phosphatase, is located in band q23 of chromosome 10, and it was found to be inactivated in some GBM cases[6]. This protein is a tumor suppressor, acting as a regulator of the cell cycle and limiting cellular growth. PTEN alterations prevent the activation of the Akt/mTOR pathway and since Akt is one of the STKs that play an essential role in cellular proliferation, the inhibition of this pathway results in the deregulation of cell cycle[4,14]. Mutations on the retinoblastoma protein (RB) gene, located on chromosome 13, are also found in glioblastoma. The RB protein, when hyperphosforilated, can block the action of transcription factors, interfering with the cell cycle[8,21]. NF-κB, is a protein complex that controls cell proliferation and cell survival by regulating DNA transcription and regulating specific genes associated with this process. PDGF overexpression promotes glioma cell proliferation by aberrant activation of NF-κB in GBM[7]. It was shown that the high levels of NF-κB may be due to the inactivation of the PI3K pathway, which has been implicated in mediating the activation of PTEN and PDGF expression[22].

Another key feature in glioblastoma is angiogenesis. Higher vascularity is correlated with high malignancy and tumor aggressiveness. Vascular endothelial growth factor (VEGF) and its receptors are involved in glioblastoma angiogenesis. VEGFs are secreted by the tumor and are able to cause vascular permeability[15,23]. VEGF/VEGFR (VEGF receptor) participates also in the formation of primitive blood vessels and in the further development of blood vessels in gliomas[21].

Necrosis occurs in astrocytomas when tumor cells achieved a high malignant state, constituting the major feature oh higher grade gliomas[7,24]. Many factors can cause necrosis, including regions of fast growing cells or vascular thrombosis. Vascular thrombosis occurs in most cases, due to the disorganized, tortuous and functionally abnormal vascular structure of GBM and can lead to tissue hypoxia and, finally, to cellular necrosis[4,20].

1.1.4) GBM Treatment

The standard treatment for Glioblastoma consist in the surgical removal of the tumor, followed by chemotherapy and radiotherapy. However, even with the help of contrast agents, it is impossible to remove all cancer cells due to the ability of GBM to infiltrate the surrounding tissue[4,21].

One of the biggest problems related with treatment of GBM is the BBB (blood brain barrier), which is a structure of brain capillary endothelial cells that regulates molecular and cellular passage to the nervous tissue. The amount and type of molecules that can reach the brain is very limited due to the tight junctions between endothelial cells and the absence of specific receptors[25] . This greatly affect the majority of drugs available for cancer treatment, which cannot cross the BBB or, do not cross in efficient concentrations, that not cause excessive toxicity to the healthy tissue. To overcome this problem, several new treatment options have been proposed, based on modulation of BBB permeability or on the use of particles capable of overcoming this barrier[25].

Temozolomide (TMZ), an oral alkylating and chemotherapeutic agent, was first used 1993 and has become a major agent for treating primary brain tumors following surgical resection and radiotherapy. It alkylates or methylates DNA, causing cancer cells to die. Nevertheless, GBMs are highly resistant to a single drug, suggesting that dual strategies involving standard chemotherapies like TMZ and pathway inhibitors might be a possible future direction for treating GBM[26,27]. For instance, TMZ together with the erlotinib, an EGFR inhibitor, and radiotherapy have recently been reported to improve patient survival[26].

Sunitinib is an orally bioavailable drug which has has been identified as an inhibitor of the angiogenic RTKs, such as the PDGFR, VEGFR-1 and VEGFR -2. The simultaneous

inhibition of these targets leads to reduced tumor vascularization and cancer cell death and, finally, to tumor reduction[28,29]. Sunitinib treatment also produced an anti-invasive effect on GBM cells[30].

New therapeutic approaches, such as immune and gene therapy also has been the target of investigation by the scientific community (figure 3).

1.1.4.1) Immunotherapy

Immunotherapy has been showing promising results in the treatment of GBM since it was discovered that tumors are immunogenic, and possess tumor specific antigens. Treatments that involve the activation of the immune system are often used, due to the immunosuppressive environment of the tumor.

Overall, there are two major ways for GBM treatment using immunotherapy. Active immunotherapy aims to boost the patient´s native immune response, while passive immunotherapy uses antibodies or activated immune cells directly targeting tumor cells[9,31].

For active immunotherapy, several antigens can be used, such as synthetic peptides, intact tumor cells and tumor protein lysates. Synthetic peptides, usually of small size, are injected as a vaccine in order to trigger an immune response in the patient by binding to MHC (Major Histocompatibility complex) class I molecules, which leads to activation of cytotoxic T lymphocytes. On the other hand, cell based immunotherapy uses antigen presenting cells activated by tumor antigens.

Passive immunotherapy, can be further divided into three different methods. First, monoclonal antibodies can be directly injected in order to interact with specific antigens. For instance, bevacizumab is an IgG1 monoclonal antibody that binds to and neutralizes the vascular endothelial growth factor (VEGF) ligand, which is a tumor-associated protein[32,33].

A second approach is based on the use of cytokines to stimulate the immune system. In this kind of passive immunotherapy cytokine stimulation with IL-2 has been studied in wide variety of cancer[32].

The third strategy involves the treatment with stimulated immune effector cells. In this kind of therapy immune cells are activated *ex vivo* before injection into the patients. Both lymphocyte-activated killer cells (LAK) and cytotoxic T lymphocytes (CTL) have been used[9].

Nevertheless, although immunotherapy is a promising therapeutic approach for gliomas, there is a need for better clinical trials to realize how far we can go with this type of treatment.

1.1.4.2) Gene therapy

Gene therapy is the introduction of nucleic acids on the cells, in order to replace a deficient gene or to modulate the expression of specific genes. This kind of therapy has been studied as a possibility for the treatment of tumors. It is important to choose the correct vector (particle that carries the nucleic acid) in order to deliver the nucleic acid to the right cells with few side effects. Synthetic vector research has focused on the use of nanoparticles. Liposomal vectors, cell penetrating peptides and polymers, for example, have been used to deliver therapeutic genes.

For the treatment of gliomas, viral vectors are usually used for the delivery of suicide and pro-apoptotic genes. One example is the use of the herpes simplex virus to deliver the timidine kinase gene, that converts the prodrug ganciclovir (GCV) into the metabolite deoxyguanosine monophosphate, resulting on the inhibition of the DNA polymerase activity[34].

Liposomal vectors have also been used to deliver therapeutic genes. These lipid-based vesicles possess many interesting characteristics which give them several as gene delivery system. For instance, they can incorporate both hydrophobic and hydrophilic drugs and their surface can be modified to incorporate ligands that confer specificity and modulate biodistribution and pharmacokinetics.

Recently, siRNAs and miRNAs have appeared in the forefront of research for the treatment of GBM. These molecules can modulate the expression of specific genes at the post-transcriptional level. The combination of miRNA regulation with gene delivery strategies allows to target and modulate the expression of endogenous genes, either by downregulation of the gene mRNA or by the silencing a specific miRNA, aiming at

upregulating its target mRNAs[35,36]. For instance, microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is downregulated in glioblastoma. Therefore, the delivery of miR-7 mimics constitutes a new approach for the disease[37].

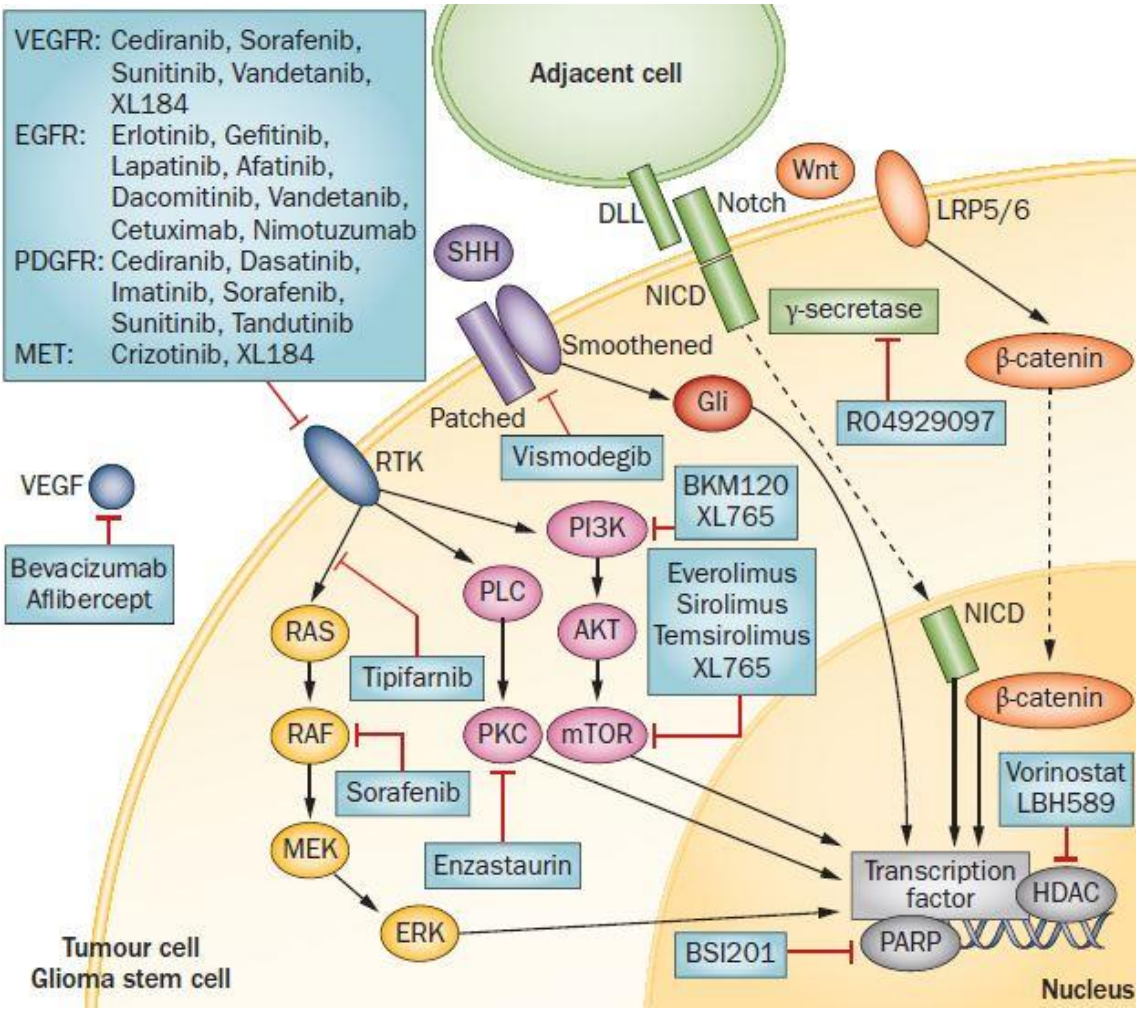


Figure 3. Therapeutic agents for glioma treatment and their molecular targets. Abbreviations: Ang, angiopoietin; bFGF, basic fibroblast growth factor; DLL, delta-like ligand; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase; FGFR, FGF receptor; HDAC, histone deacetylase; HGF, hepatocyte growth factor; JAK, Janus kinase; LRP, lipoprotein receptor-related protein; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; NICD, Notch intracellular domain; PARP, poly(ADP-ribose) polymerase; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PLC, protein lipase C; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; RTK, receptor tyrosine kinase; SHH, sonic hedgehog; STAT, signal transducers and activators of transcription; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor. Adapted from[121]

1.2) Cancer Stem Cells

Stem cells are a group of undifferentiated cells with special functions that occur in a large variety of somatic tissues. These cells are able to differentiate, self-renewal and control cellular homeostasis. They can form identical stem cells with the same potential for differentiation, thus maintaining the stem cell pool, or originate new cellular types that loose these characteristics Within the tumor, there are a minority of cells that share some characteristics with stem cells, which are called the Cancer Stem Cells (CSCs)[38,39].

The first evidence for CSCs came from myeloid leukaemia, where a group of researchers was able to induce leukaemia following transplantation of these cells. CSCs have the capacity to self-renewal and are able to generate the different type of cells that comprise the tumor, sustaining tumorigenesis[40]. Some results show that this types of cells are more resistant to radiotherapy and chemotherapy. The existence of these cells could be one of the reasons for the heterogeneity of the tumors since they can undergo aberrant differentiation to many different cell types[41]. There are four characteristics that are often associated with CSCs. First, is the fact that only a small portion of cancer cells has the ability to perform tumorigenesis when transplanted into immunodeficient mice[40]. In addition, these cells have specific surface markers that can be used to promote their isolation by immunoselection. Moreover, the tumors generated from CSCs contain both tumorigenic and non-tumorigenic cells. Finally, CSCs can be transplanted through many generations, maintaining their self-renewal capacity[39,42,43].

There is one hypothesis that states that CSCs self-renewal and differentiation are maintained by the division of one stem cell in two different daughter cells, one similar to the parental cell and another that will undergo differentiation. There are some well-known self-renewal regulators, such as the transcriptional repressor Bmi-1 and Wnt/-catenin signaling pathway of the polycomb family, that have been shown to be involved in this process[11,13].

1.2.1) Origin of CSCs

It is accepted by most scientists in the field that CSCs are formed by mutated (epigenetic and genetic modifications) stem cells or progenitor cells of some organs that subsequently grow and differentiate to create primary tumors (Figure 4), but this area continues under

research. There are also some evidence of formation of CSCs from cells recruited from other organs[11,43].

Alteration of self-renewal pathways seems to be an important mechanism underlying CSCs formation. For instance, BMI-1, a transcriptional repressor and Wnt/β-catenin pathways, seems to be involved in the acquiring of self-renewal capacity by CSCs[44].

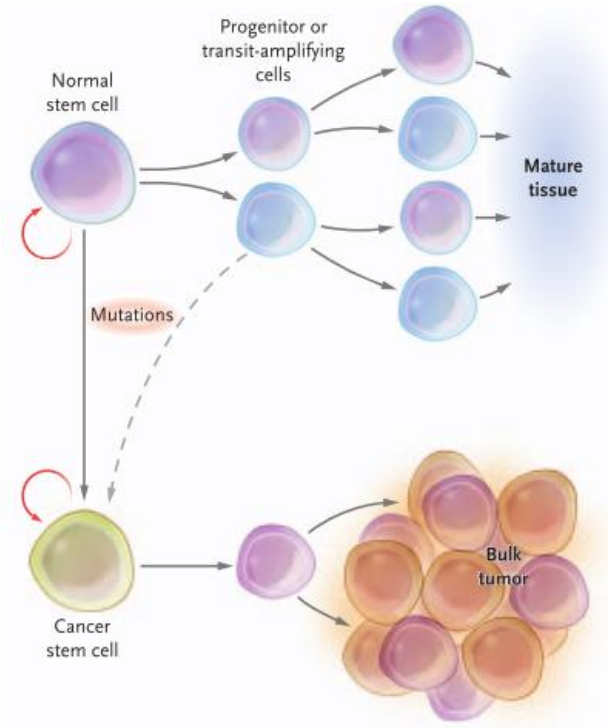


Figure 4 – Possible mechanism for the formation of cancer stem cells. Stem cells have the ability to self-renewal and differentiate. When normal stem cells suffer mutations, they can originate a specific type of stem cells, the cancer stem cells. Adapted from [122]

1.2.2) Self-Renewal and Differentiation Pathways

It is well known that CSC have the ability to form new stem cells and maintain an intact potential for proliferation, expansion, and differentiation, thus the stem cell pool[45]. Molecular pathways that are important for CSCs biology are described below and summarized in table 2.

The Wnt/β-catenin pathway induces proliferation of progenitor cells within gliomas and other types of tumors. The canonical Wnt cascade is one of critical regulators in stem cells. Recent studies identified the Wnt/β-catenin self-renewal pathway as an important

pathway for the maintenance of several CSC, such as breast CSCs. The observation of the overexpression of Wnt3a and Wnt1, Wnt ligands, in CSC supports the hypothesis that this pathway is important for CSC self-renewal and radioresistance[46].

The Sonic Hedgehog (SHH) pathway is a key regulatory pathway critical for the maintenance of several types of cells, including neural stem cells. Sonic Hedgehog signaling begins with the binding of Hedgehog ligands to the PTCH (Protein patched homolog 1) receptor. With this binding, gliotactin (Gli) signal transducers are activated and then translocated to the nucleus, where they regulate the transcription. This protein shown to contribute to the self-renewal and tumorigenic potential of CSCs, whereas its blockage leads to apoptosis and inhibition of migration[43,45].

Notch pathway is known to play an important role in CSC growth and differentiation. The Notch family of transmembrane receptors proteins comprise four members (Notch 1–4). These receptors mediate cellular processes through the interaction with ligands (Jagged-1,-2, and Delta-like-1, -3, and-4). Notch-signaling is essential for the maintenance of somatic stem and progenitor cells by supporting self-renewal and suppressing differentiation[43]. Using γ-secretase, inhibitor of Notch pathway, it was possible to demonstrate the impairment of cell growth, clonogenic survival and tumor formation ability. Although highly important for self-renewal, some studies also suggest that Notch signaling is important for differentiation of CSCs into tumor-derived endothelium[42,47].

The PI3K/AKT/ pathway signaling pathway is involved in CSC biology, mainly on cell cycle progression and survival. AKT negatively regulates glycogen synthase kinase-3β (GSK-3β), promoting β-catenin-induced stem cell self-renewal. In some cancer types, such as breast cancer inhibition of the AKT pathway reduced CSC effectiveness[43].

Signal transducer and activator of transcription 3 (STAT3) activation is essential for stem cell differentiation and survival. STATs can be phosphorylated by activated tyrosine kinase receptors, resulting in the formation of homo- and heterodimers that enter the nucleus and alter gene transcription. Based on inhibition strategies of STAT3 pathway using curcubitactin 1, researchers were able to differentiate $CD133^{+}$ cells into $CD133^{-}$ cancer negative cells[41].

BMP (bone morphogenic protein) has an important role on differentiation signal on several cancer types, including GBM. The use of BMP4, an inhibitor of BMP signaling, led to a differentiation and proliferation block[43].

Table 2 – Overview of molecular pathways involved in CSC

Pathway	Cancer	Function	Ref.
WNT	Breast CML AML	Involved in self-renewal, maintenance and radioresistance of cancer stem cells.	42,44,46
Sonic Hedgehog	Breast Glioblastoma CML Colon	Promotes self-renewal, migration and tumorigenesis.	44,48,49
Notch	Colon Breast Glioblastoma	Important in the maintenance of CSC and tumorigenesis. Recently has been reported to be involved in differentiation.	42,43,50
BMP	Glioblastoma	Inhibition of asymmetric division.	7,10,43
STAT	Glioblastoma Pancreas Breast	Essential for stem cell differentiation and survival.	13,51
PI3K/AKT	Prostate Pancreas Glioblastoma	Promotion of GSC self-renewal. Proliferation and survival of GSCs. Tumorigenesis.	43,52
TGF-β	Glioblastoma	CSC initiation and maintenance.	22,45

1.2.3) Resistance Mechanisms

It is common knowledge that CSCs are more resistant to radiotherapy and chemotherapy compared to normal cancer cells, which allows them to remain in the tissue leading to tumor reappearance even after treatment[50]. Although the mechanisms for the development of cancer stem cell resistance still need to be studied in more detail. It is known that enhanced DNA damage response (DDR), activation of self-renewal pathways and overexpression of ABC transporters play an important role in CSC resistance to therapies[12,13,53].

In glioblastoma, it has been shown that $CD133^+$ cells are able to respond to radiation damage more efficiently and undergo less apoptosis when compared with $CD133^-$ cells[54].

The reaction to DNA damage caused by irradiation comprises several kinases, such as the CHK1 and CHK2. Activation of CHK1 initiates cell cycle DNA repair and cell death to prevent damaged cells from progressing through the cell cycle, while CHK2 is a cell cycle checkpoint regulator and a tumor suppressor. These results are strengthened by the fact that CSCs can be sensitized by inhibition of this two kinases. Similar results were observed with inhibition of TGFβ and ALDH1 pathways, suggesting that these pathways can be also involved on CSC resistance[13].

In addition, the adenosine triphosphate-binding cassette (ABC) transporters can act as drug efflux pumps, working as protectors of many cell types, including CSCs. These cells can be sensitized by ABC transport inhibitors, such as the verapamil[13].

Recent studies have also suggested that Wnt and β-catenin signaling may contribute to radioresistance of cancer stem cells[13].

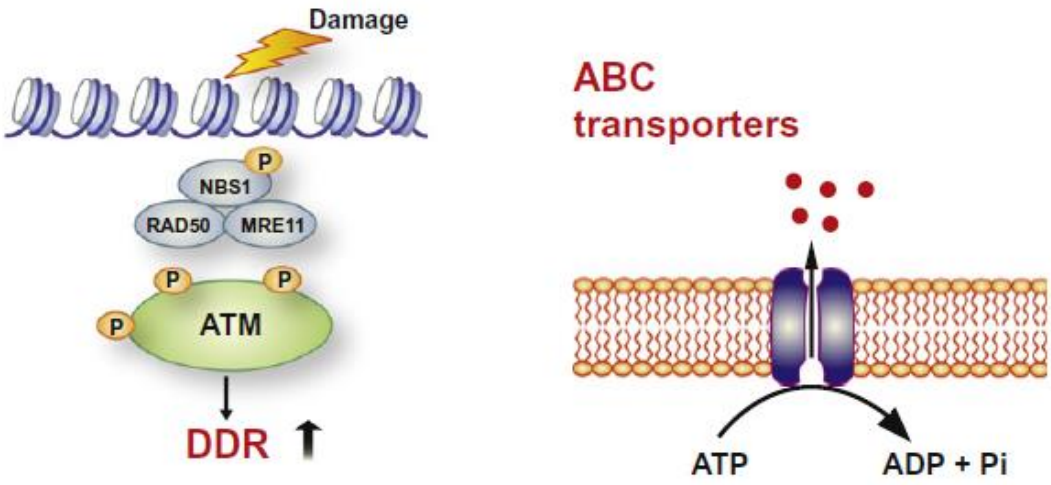


Figure 5 – Mechanisms of CSCs resistance to therapy. Enhanced DNA damage response (DDR) can be observed after irradiation in CSCs. High levels of ABC transporters are often associated with tumor resistance to therapy. Adapted from [13]

1.2.3.1) Therapeutic Strategies for Cancer Stem Cells

Therapies that target specifically CSCs in order to eradicate the tumor are essential due to its self-renewal and tumorigenic properties, thus is important to evaluate the differences between CSCs and normal cancer cells**.** Current strategies target the bulk of the tumor and do not eradicate CSC completely, which is essential for the cure of the cancer since

CSC are implicated in the development of therapy resistance (figure 5) and in tumor recurrence[13,42].

Since CSC are rare among the tumors, the recognition of CSC within the tumor is the first challenge. It is necessary to identify specific antigens within CSCs, and because CSC of the different tumors have come from different origins, to develop therapeutic strategies targeting different CSC populations[42].

One of the strategies for CSC treatment consists in the specific eradication of CSC preventing the tumor to reoccur. Ideally, in this strategy it is needed to target pathways uniquely used by cancer stem cells to generate the cancer cells.

Another treatment strategy relies in the targeting of the pathways involved in CSC-mediate resistance to therapies. For instance, CSC can be sensitized to irradiation by inhibition of Chk1 and Chk2, which are essential for DNA repair. TGFβR-1 kinase inhibitor is also able to enhance sensitivity to drugs, since TGFβ plays an important role in glioblastoma CSC resistance[13].

Differentiation therapy is based on the induction of CSC differentiation to make tumor growth unsustainable. For instance, differentiation of these cells can be induced by all-trans retinoic acid (ATRA), associated with Notch pathway down-regulation or, alternatively, it can be achieved by modulating miRs that also target the Notch pathway in glioblastoma, such as miR-34a, miR-124 and miR-137[13,55].

Inhibition of ABC transporters, which are transporters responsible for drug efflux is also an available therapeutic option. High levels of ABC transporters are often associated with poor prognosis, suggesting that these transporters are essential for tumor resistance to therapies[13].

1.2.4) Markers

Being hierarchically distinct populations, CSCs populations can be easily isolated via the expression of specific surface markers. Table 3 show some well-known CSCs markers for various types of tumors, such as the ubiquitous aldehyde dehydrogenase (ALDH1), CD133 (prominin 1), CD44 and nestin.

Many researches succeeded on the isolation of CSCs from glioblastoma using ALDH1, CD133 and CD44 as molecular markers. ALDH1 catalyzes the oxidation of aldehydes to carboxylic acids, having an important role in proliferation and migration.

CD133, also known as proiminin 1, is a transmembrane glycoprotein. This protein is usually found in CSCs of glioblastoma, being the most used cell surface marker for the isolation of these cells, it was shown that knockdown of CD133 impairs self-renewal of CSCs, suggesting that this protein may be involved in this mechanism[42].

CD44, which is also a surface glycoprotein, is involved in cellular adhesion and migration and is the receptor for hyalunoran-mediated motility[19,56].

Despite their frequent use for CSC isolation, these markers have some associated problems. For instance, a single CSC marker may not be specific on its own and may need to be combined with at least a second markers to achieve good results. Another common problem is that markers can be valid for one separation method (for example, fluorescence-activated cell sorting), but not in others (for example, immunohistochemistry)[57]. Nevertheless, and despite the fact that none of this markers is universal for all cancer types, they provide good results in the isolation of cancer stem cells from different kinds or tumors.

Table 3- Cancer stem cells specific markers in the different cancer types.

Glioma	Colon	Breast	Lung	Liver	Ovarian
CD15	ABCB5	ALDH1	ABCG2	CD13	CD24
CD90	ALDH1	CD24	ALDH1	CD24	CD44
CD133	CD24	CD44	CD90	CD44	CD117
Nestin	CD26	CD90	CD117	CD90	CD133
	CD29	CD133	CD133	CD133	
	CD44				
	CD133				
43,53,57	57,58	43,57,58	58,59	57	57
References					

1.2.5) Role of CSCs in Glioblastoma Multiforme

Glioblastoma multiforme is a highly aggressive and invasive tumor that displays extreme resistance to radiotherapy and chemotherapy and has a high rate of recurrence. Some of these characteristics are due to the presence of Glioma stem cells (GSCs), a group of cells that, similarly to other CSCs, is highly resistance to therapy and presents high capacity of self-renewal. These cells also share some properties with normal neural stem cells, such as the enhance potential for proliferation, angiogenesis and invasion. GSCs remains controversial because of unresolved questions related with the frequency of these cells, the surface markers by which they can be identified/isolated, and the nature/origin of these cells.

The first evidence for GSCs came from Dirks and colleagues, who isolated cells from human GBM samples based on expression of the cell surface glycoprotein CD133 (Prominin1/PROM1)[60]. Until today, and despite all referred drawbacks, CD133 is still considered the universal marker for CSC in glioblastoma. Paolo Brescia and colleagues demonstrated that CD133 is not only a marker for CSC, but it is also involved in the maintenance of the tumorigenic potential of GBM stem cells. By silencing CD133, they obtained a reduction of growth, self-renewal and the tumor-initiating ability of these cells. These results suggest that targeting $CD133^{+}$ cells could be an interesting therapeutic approach[54,61,62].

In addition, GSCs were shown to have increased expression of nestin, an intermediate filament protein expressed in neural stem cells. The hallmarks of $Nestin^{+}$ cells are proliferation, migration and a broad differentiation potential[10,63,64].

Many researches have shown that GCSs contribute to therapeutic resistance and, as a consequence, to GBM recurrence. By measuring the activating phosphorylation of several critical checkpoint proteins in DNA response (ATM, Rad17, Chk2 and Chk1) Bao and colleagues demonstrate that GCS are more resistant to radiation when compared to the non-stem glioma cells[10]. GCSs can be sensitized to radiotherapy with γ-secretase, a notch pathways inhibitor, suggesting that this pathway plays a role on GCS resistance[10].

Strong angiogenic activity is another of the major hallmarks of glioblastoma where GSCs seem to be involved. High expression of pro-angiogenic factor, vascular endothelial growth factor (VEGF), found in GCS, suggests that these cells play a role in angiogenic processes associated with glioblastoma[10,15].

Hypoxia, another hallmark of glioblastoma, increases the expression of GSC markers and self-renewal indicators, suggesting that the cancer stem cell-like phenotype can be promoted by the micro-environment conditions found in the tumors. Focusing on the hypoxic niches, disrupting the GCS microenvironment can be a new approach for therapeutic strategies focusing GCSs[13,65,66].

1.3) miRNAs

Gene expression is a complex process by which the information from a gene is translated into the synthesis of a functional gene product, usually a protein. Along this biological process, regulators of gene transcription and translation operate at multiple levels in order to optimize the genome end products. One of the most significant advances in gene regulation has been the discovery of small (20–30 nucleotides) noncoding RNAs that regulate genes and genomes. This regulation can occur at the level of chromatin structure, chromosome segregation, transcription, RNA processing, RNA stability and translation[67–69]. Different classes of small RNAs have emerged and can be categorized in three major types: short interfering RNAs (siRNAs), microRNAs (miRNAs), and piwi-interacting RNAs (piRNAs)[69].

SiRNAs, a class of double-stranded RNA, are involved in the RNA interference pathway, where they interfere with the expression of specific genes to which they present complementary nucleotide sequences. SiRNAs cause mRNA to be degraded after transcription, therefore preventing protein synthesis[67].

MicroRNAs (miRNAs) are small noncoding RNAs with ~21–23 nucleotides that act as regulators of gene expression in multicellular eukaryotes. These small RNA molecules were discovered for the first time in 1993 in *Caenorhabditis elegans* by Lee et al., and are now described to be involved in many cellular processes such as the regulation of signaling pathways, apoptosis, metabolism and brain development. MicroRNAs enhance the cleavage or translational repression of specific mRNAs that contain miRNA binding site(s) in their 3'untranslated region (3′UTR). Some studies indicate that miRNAs can control most of the protein-coding genes, being involved in almost every biological pathway[67–69]. Therefore, deregulation of miRNAs is described to play and important role in many diseases, including cancer[68].

1.3.1) Biogenesis

MicroRNA loci are located in intronic regions of protein-coding and noncoding genes and also in exons of long ncRNA (non-coding RNA) transcripts[70]. Starting from the chromosome, miRNA synthesis is highly regulated from the nucleus to the cytoplasm to.

MicroRNA biogenesis proceeds according to has two major pathways: canonical and non-canonical [71](figure 6).

1.3.1.1) Canonical Pathway

Most mammalian miRNAs are transcribed from the genome by RNA polymerase II, generating a primary miRNA (pri-miRNA) transcript that consists of one or more hairpin structure[72,73]. These pri-miRNAs are enclosed in introns of RNA polymerase II transcripts (intronic miRNAs) or can be transcribed from independent miRNA genes (exonic miRNAs). Pri-miRNAs can be polyadenylated and caped after transcription. After transcription, pri-miRNAs are processed by Drosha (an RNase III enzyme present in the nucleus) and by the dsRNA-binding protein DGCR8 (also known as Pasha in invertebrates). The resulting product of this processing is a molecule of RNA with 70 nucleotides called pre-miRNA. Pre-miRNAs are transported to the cytoplasm by exportin 5, in a GTP-dependent process. In the cytoplasm, pre-miRNAs are cleaved by endonuclease DICER and the RNA-binding protein TAR (TRBP)[74,75]. After processing by the DICER/TRBP protein complex, the resulting product is one hairpin structure with 20-23 nucleotides. Following their processing, miRNAs are assembled into ribonucleoprotein (RNP) complexes called micro-RNPs (miRNPs) or miRNA-induced silencing complexes (miRISCs)[72,73]. The key components of miRNPs are proteins of the Argonaute (AGO) family. In mammals, four argonaute proteins have been characterized (AGO1 to AGO4)[75].

1.3.1.2) Non-Canonical Pathway

Drosha mediated processing of pri-miRNAs into pre-miRNAs is not obligatory**.** In the non-canonical pathway, discovered and characterized in 2007 by Sibley and colleagues, miRNA precursors are produced via splicing and are called mirtrons[76]. These RNA molecules are splicing-produced short-hairpin introns with equivalent hallmarks of pre-miRNAs. Mirtrons are transported to the cytoplasm by exportin 5 in a similar process to that occurring in the canonical pathway[76]. Due to the similar characteristics of mirtrons and pre-miRNAs, mirtrons are able to enter the canonical miRNA-processing pathway[73].

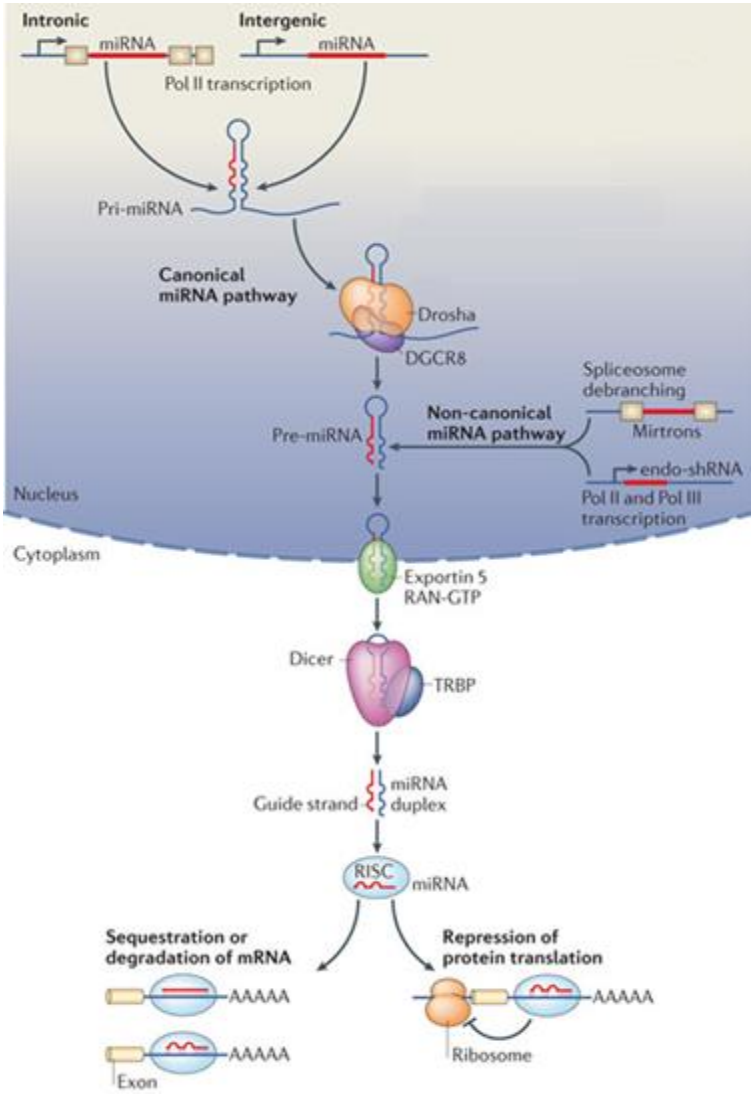


Figure 6. **Biogenesis of miroRNAs and their assembly into microribonucleoproteins.** The canonical pathway starts with the production of precursor miRNAs (pre-miRNAs) by Drosha-mediated cleavage of primary miRNA transcripts (pri-miRNA). The non-canonical pathway, starts with the production of pre-miRNAs by splicing-mediated cleavage of short-hairpin introns (mirtrons). After their processing, miRNAs are assembled into ribonucleoprotein (RNP) complexes (miRNPs) or miRNA-induced silencing complexes (miRISCs). The key components of miRNPs are proteins of the Argonaute (AGO) family. In mammals, four AGO proteins (AGO1 to AGO4) function in the miRNA repression pathway, but only AGO2 functions in RNAi pathway and leads to direct mRNA cleavage. DGCR8: DiGeorge syndrome criticical region gene 8 protein; TRBP: RNA-binding protein TAR; Adapted from [72]

1.3.2) MicroRNA Mechanisms for Translational Repression

Gene silencing by miRNAs may occur either via mRNA degradation or translation blockage. Protein levels of the target gene are consequently reduced, whereas messenger RNA levels may or may not be decreased[77].

Despite the imperfect pairing of miRNAs with their targets, there is a region of perfect base pairing comprising the nucleotides 2–8 of the miRNA. This regions represents the 'seed' region, which is essential for the miRNA/mRNA interaction. MicroRNA-binding sites in mRNAs are located in the 3′ UTR and are usually present in multiple copies. A high degree of complementarity between miRNAs and sequences on the 3' UTR of the target mRNA is essential for gene silencing mediated by miRNAs[70,78].

Initiation, elongation and termination are the three steps of mRNA translation. Initiation starts with the recognition of the mRNA 5′-end and its cap structure (7-methylguanosine, m7GpppN) by the eIF4E subunit of the eukaryotic translation initiation factor (eIF) eIF4E[72]. This initiation factor contains eIF4G, which is essential for the assembly of the ribosome initiation complex. EIF4G, with the help of eIF3, facilitates the recruitment of the 40S ribosomal subunit to mRNA. The 60S subunit is then attached to the small subunit to start mRNA translation. There is substantial evidence that suggest that miRNPs interfere with the eIF4E–eIF4G interaction, which prevents the assembly of the 40S initiation complex. An alternative theory suggests that miRNPs are able to repress translation by preventing 60S subunit from joining 40S[74,77].

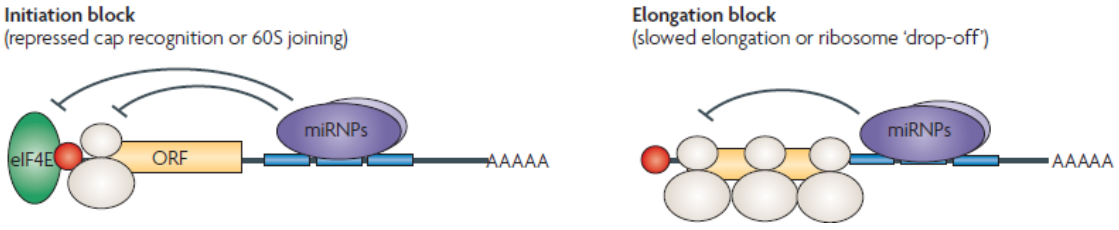


Figure7. Mechanisms of miRNA-mediated inhibition of protein translation in animals. MiRNP-mediated translational repression can occur at either initiation or post-initiation steps. The miRNP complex inhibits translation initiation by either interfering with 5' cap (m7G) recognition and 40S small ribosomal subunit recruitment or antagonizing 60S subunit joining and preventing 80S ribosomal complex formation. Additionally, the miRNP complex inhibits translation at post-initiation steps by inhibiting ribosome elongation. ORF: Open reading frame; eIF4E: eukaryotic translation initiation factor (eIF) eIF4E; miRNPs: ribonucleoprotein complexes. Adapted from [72]

The mechanism by which miRNAs repress translation does not focus exclusively in the initiation step. Several theories state that MiRNAs can also repress mRNA translation at the post-initiation steps. For example, MicroRNAs might slow the process of elongation, promote the degradation of the polypeptide or cause the detachment of the ribosomes during the process of translation[70].

1.3.3) Biology of miRNAs in Gliomas

Most cellular processes are affected by miRNAs. In invertebrates, miRNAs regulate development, neuronal differentiation, cell proliferation, growth control, and apoptosis. In mammals, miRNAs have are important for embryogenesis and stem cell maintenance, hematopoietic cell differentiation and brain development. In most human diseases, including cancer, miRNA expression has been found to be deregulated, suggesting that these small RNA molecules may be involved is these syndromes[68,79]. Malignant tumors and tumor cell lines were found to have widespread deregulated miRNA expression compared to normal cells. However, in most cases it is not clear whether the altered miRNA expression observed in cancer is a cause or consequence of malignant transformation[77].

Many studies identified the importance of miRNAs in human glioma, where a significant number of miRNAs have been found to be deregulated and contribute to disease development and progression. MicroRNAs modulate most glioma cellular functions such as proliferation, invasion, migration, angiogenesis, resistance to therapy and apoptosis[42,80]. Table 4 shows several miRNAs that are deregulated in GBM, as well as some of their validated targets.

1.3.3.1) MicroRNAs altered in Gliomas and their role on Gliomagenesis and Glioma Stem Cells

Global analysis of miRNA expression profiles in glioblastoma cell lines allowed to identify miRNAs with significantly altered expression in this type of tumor and which contribute to making it more aggressive and proliferative[81–83].

In this regard, miR-137 (downregulated in glioblastoma) targets and suppresses CDK6 expression, a positive mediator of cell cycle progression. Its downregulation enhances glioma cell proliferation, and lower miR-137 levels are associated with a poorer

prognosis. Studies using glioblastoma cell lines, showed that transfection of mic roRNA-137 also induced G1 cell cycle arrest, suggesting that this miRNA´s downregulation in glioblastoma could be important for its active proliferation[84].

MicroRNA-34a, also downregulated in gliomas, targets the mRNAs of multiple growth-promoting genes, including E2F transcription factor 1 (E2F1), hepatocyte growth factor receptor (c-met), and CCND1. These proteins are important for sustaining the growth of glioma cells, and since miRNA-34a will repress their translation, the control of the tumor growth will be impaired[85]. Recently, it was also shown for the first time that miR-34a expression induces glioma stem cell differentiation. In the study, transfection of miR-34a into glioma cells led to a decrease in the immunostaining of stem cell markers CD133 and nestin[86].

Two other microRNAs involved in GBM, miR-181 and miR-153 promote apoptosis by targeting B-cell chronic lymphocytic leukemia/lymphoma 2 (Bcl-2) mRNA and repressing its translation, thus inhibiting gliomagenesis. Both miR-181 and miR-153 expression is decreased in glioma cell lines, suggesting that these two miRNAs have an important role in glioma by diminishing its programmed cellular death[87].

MicroRNA-128 is another well-known miRNA downregulated in glioblastoma. This miRNA has multiple targets of interest, including E2F3a, a transcription factor that induces the expression of genes involved in cell cycle progression, and Bmi-1, a member of the polycomb repressor complex (PRC1) involved in stem cell renewal[85]. BMI, a protein involved in stem cell self-renewal, was the first validated target for miRNA-128[88]. Upon miR-128 induction, this protein was found to be downregulated. Xiaozhong Peng and colleagues, using a luciferase reported assay, showed that E2F3a was negatively regulated by miR-128. This results present strong evidence that miR-128 can inhibit the proliferation of glioma cells through negatively regulating one of its targets, E2F3a, which is highly expressed in glioma and important for cell cycle progression[89]. More recently, a group of researchers showed that MicroRNA-128 coordinately targets polycomb repressor complexes (PRC) in glioma stem cells[90]. The Polycomb Repressor Complex (PRC), an epigenetic regulator of transcription, is mediated by 2 protein complexes, PRC1 and PRC2. This complex has high oncogenic potential in glioblastoma, where it is involved in cancer stem cell maintenance and radioresistance. In this study, the authors showed that miR-128 simultaneously targets important constituents of PRC 1

and 2 and that its downregulation in glioblastoma contributes to a high level of expression of these proteins compared with normal brain cells. In addition, miR-128 expression increases radiosensitivity of GSCs by preventing the radiation-induced increase of expression of PRC components, possibly by impairing DNA repair[90].

MiR-7 is an intronic miRNA, also downregulated in gliomas, which targets EGFR, a receptor known to be upregulated in 45% of malignant gliomas. Besides EGFR, recent studies showed that miRNA-7 also targets IRS-1 and IRS-2, two important regulators of the AKT pathway[91]. Moreover, transfection with miR-7 oligonucleotides was shown to decreased the viability and invasiveness of primary glioblastoma cell lines[37].

Contrarily to the above mentioned miRNAs, miR-10b, which is highly expressed in a number of cancers and has an important role in tumor growth and metastasis, was found to be upregulated in GBM. MicroR-10b inhibits the translation of the mRNA encoding HOXD10, which modulates many genes that promote invasion, migration, extracellular matrix remodeling and tumor progression, including uPAR, RhoC, integrin, βintegrin and matrix metalloprotease-14 (MMP-14)[92]. Recent studies have found that inhibiting the expression of miR-10b reduces GBM cell growth and significantly decreases GSC proliferation, migration and invasion[93].

MicroRNA-221 and miRNA-222, also upregulated in glioblastoma, have been reported to regulate cell growth and cell cycle progression by targeting p27 and p57[80]. In their study in 2010, Chun-Sheng Kang and colleagues demonstrated for the first time that miR-221/222 directly regulate apoptosis in glioblastoma by targeting PUMA. These miRNAs negatively regulate PUMA, which leads to a decrease in anti-apoptotic Bcl2 and to an increase in pro-apoptotic BAX[94].

MiR-21, which is the most studied miRNA in glioma, has been consistently reported to be upregulated in these tumors. The validated targets of miR-21 include p53, a tumor suppressor protein, and TGF-β, a protein that controls cellular proliferation and differentiation [95,96]. MicroRNA-21 also promotes glioma invasion by targeting matrix metalloproteinase regulators, such as the RECK, a membrane-anchored regulator, and TIMP3, the ECM-bound protease regulator[97]. These targets suggest that miR-21 has oncogenic potential, negatively regulating tumor suppressor functions.

MicroRNA-221 and miRNA-222, also upregulated in glioblastoma, have been reported to regulate cell growth and cell cycle progression by targeting p27 and p57[80]. In their

study in 2010, Chun-Sheng Kang and colleagues demonstrated for the first time that miR-221/222 directly regulate apoptosis in glioblastoma by targeting PUMA. These miRNAs negatively regulate PUMA, which leads to a decrease in anti-apoptotic Bcl2 and to an increase in pro-apoptotic BAX[94].

MicroRNA-26a suppresses PTEN, RB1 and MAP3K2/MEKK2 expression[98]. In 2013, Bing-Hua Jiang and colleagues showed that miR-26a directly targeted prohibitin (PHB) in glioma cell lines. This protein has been implicated in the regulation of proliferation, apoptosis, transcription and mitochondrial protein folding[99]. In their study, the authors present evidence that miR-26a regulates PHB and promotes glioma progression and angiogenesis[100].

MicroRNA-451 has also been found to be overexpressed in GBM cells and may function as an oncogene. MiRNA-451 modulates the AMPK pathway by controlling expression of its upstream activator, LKB1, via direct regulation of CAB39 expression [85,101]

In conclusion, over the past years, a large number of studies has suggested that miRNAs can play important roles in the development of malignant gliomas. Figure 8 summarizes the major miRNA-targeted approaches evaluated so far for GBM**.** These small RNA molecules may have their expression deregulated during tumor development and progression, which makes them interesting molecules to explore as potential diagnostic and prognostic biomarkers. In addition, the development of glioma-directed therapies based on miRNAs is also a promising field, posed to have a huge impact in healthcare, if the challenges common to all gene therapy approaches can be overcome[80,87]

Table 4 - MiRNAs deregulated in glioblastoma and their verified targets

MicroRNA	Regulation	Targets	References
miR-7	Downregulated	EGFR, IRS-1, IRS-2	35,37,91
miR-10b	Upregulated	HOXD10, MMP-14	35,91,93,102
miR-21	Upregulated	p53, TGF-β, RECK, TIMP3	35,86,103,104
miR-34a	Downregulated	E2F1, CCND1, c-MET, CDK6	80,85,91
miR-26a	Upregulated	PTEN, RB1, MAP3K2/MEKK2 PHB	35,98,100
miR-128	Downregulated	E2F3a, PRC, BMI	85,89,105,106
miR-137	Downregulated	CDK6	84,91,107
miR-153	Downregulated	Bcl-2	79,88,91
miR-181	Downregulated	Bcl-2	91,106
miR-221/222	Upregulated	p27, p57, PUMA	94
miR-451	Upregulated	CAB39, PI3K/Akt	101,108

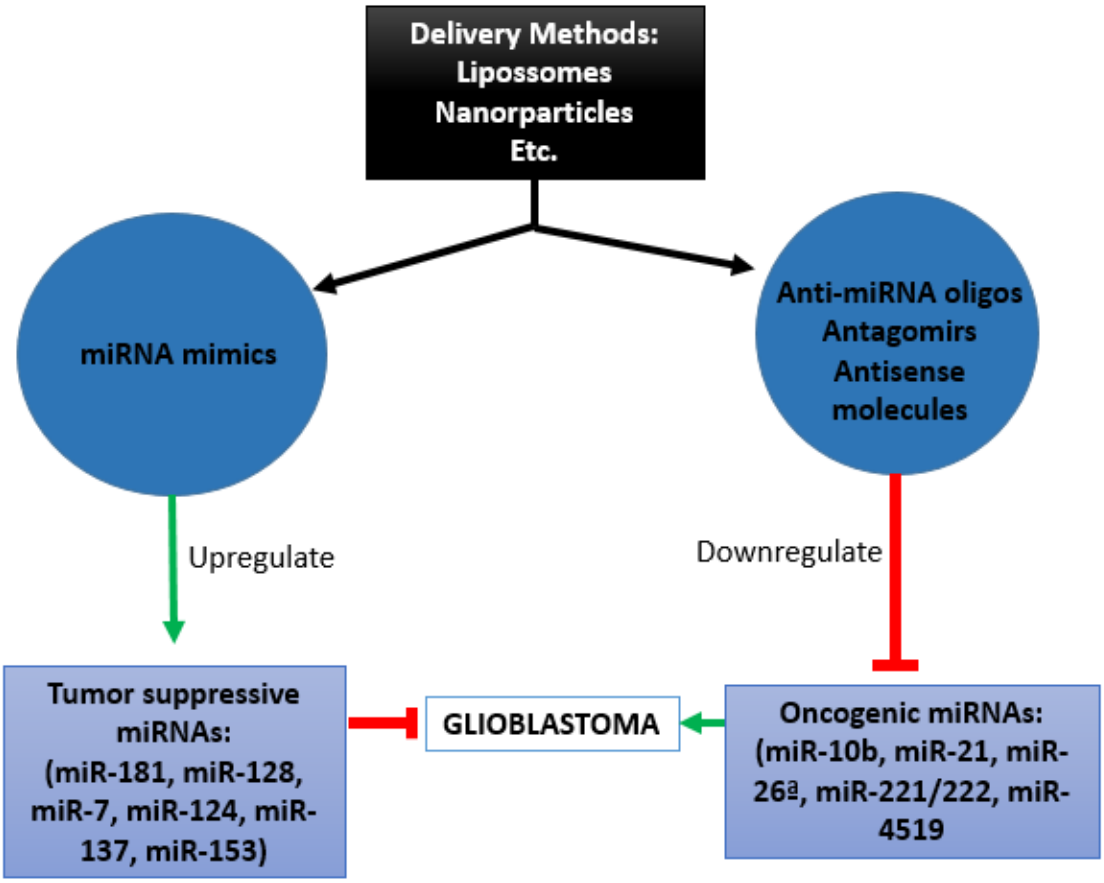


Figure 8. MiRNA-targeted therapies in GBM. **Figure 8. MiRNA-targeted therapies in GBM.** MiRNA-based therapeutic approaches for glioblastoma include the delivery, using different kinds of nanosystems, of miRNA mimics, designed to upregulate certain tumor suppressor miRNAs or anti-miRNA oligonucleotides, such as antagomiRs, antisense molecules or miRNA masks, developed to downregulate specific oncogenic miRNAs.

Chapter 2 Objectives

2) Objectives

The major objectives of this work were:

- To isolate and characterize cancer stem cells from the human glioblastoma cell line U87.
- To understand the role of cancer stem cells on the maintenance and growth of glioblastoma multiforme.
- To evaluate and compare the miRNA profile of glioblastoma stem cells with respect to differentiated glioblastoma tumor cells.
- To evaluate the role of specific miRNAs, particularly deregulated in glioblastoma stem cells, in tumor cell viability and resistance.
- To evaluate the therapeutic potential of miRNA modulation strategies, alone or in combination with the drug sunitinib, in tumor cell proliferation and viability.
- To assess the possibility of glioblastoma stem cell transfection using targeted lipid-based nucleic acid delivery systems.

Chapter 3 Materials and Methods

3.1) Materials

Sunitinib was kindly donated by Pfizer (Basel, Switzerland). Stock solutions were prepared in DMSO (Sigma, Germany) and stored at -20ºC. Custom-designed miRNA PCR plates (Pick&Mix miRNA PCR panels) were acquired from Exiqon. Primers for miRNA-128 and controls were acquired from Exiqon. CD133 human MicroBeads Kit was acquired from Miltenyi Biotec (Madrid, Spain). Lipofectamine RNAiMAX was acquired from Invitrogen. The list of antibodies used is shown in table 5.

Table 5 – List of antibodies.

Antibody	Company
Alexa-488	Life Technologies
Nestin	Sigma (N5413)
CD133-PE	Miltenyi Biotec
CD133	Enogene (E10-30240).

3.2) Cell lines and culture conditions

The U87 human glioma cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose (Invitrogen, Carlsbad, CA, USA) and supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Paisley, Scotland), 100 µg/mL streptomycin (Sigma), 100 U/mL penicillin (Sigma) and 10 mM HEPES. The cells were cultured at 37°C under a humidified atmosphere containing 5% CO2. Cancer stem cells were maintained in DMEM/F12 supplemented with B27 1x and 0.02 µg/mL FGF/EGF.

3.3) Isolation of $CD133^+$ cells

Cells were dissociated and ressuspended in PBS containing 0.5% bovine serum albumin and 2 mmol/L EDTA. For magnetic labeling, CD133/1 microbeads were used (Miltenyi Biotech). Microbeads were incubated with a maximum of 12.5 million cells for 30 min before magnetic separation (10µL of beads per 10^6 cells). Positive magnetic cell

separation (MACS) was done using several MACS columns in series. During the process, cells within the columns were washed three times, and were finally eluted after removal from the magnetic field. After isolation, $CD133^+$ cells were maintained in DMEM/F12 in a non-adherent environment, supplemented with B27 1x and 0.02 µg/mL FGF/EGF. $Cd133^-$ cells were maintained in DMEM.

3.4) Evaluation of cell viability

In the different experiments, cell viability was measured using the Alamar Blue assay. Briefly, 24 h after transfection U87/$CD133^+$ cells were incubated with DMEM containing 10% (v/v) of resazurin (Sigma, Munich, Germany). The absorbance of the medium was measured at 570 and 600 nm following 1 h of incubation at 37ºC. Cell viability was calculated as a percentage of non-transfected control cells using equation 1.

$$cell\ viabilty\ (\%\ of\ control) = \frac{[Abs_{570nm} - Abs_{600nm}]}{[Abs^*_{570nm} - Abs^*_{600nm}]} \times 100 \qquad \text{(Equation 1)}$$

ABS_{570} and ABS_{600} are the absorbance of the transfected cells, and ABS^*_{570} and ABS^*_{600} correspond to the absorbance of control cells at the indicated wavelengths.

3.5) RNAi-Lipofectamine RNAiMAX complexes preparation and cell transplantation.

For cellular transfection, we used Lipofectamine RNAiMAX (Invitrogen) according to the instructions provided by the manufacturer. For adherent cells, one day before transfection, cells were plated in 24-well plates with 500 µl of DMEM. On the day of transfection (50% cellular confluence), we prepared miRNA mimic duplex-Lipofectamine RNAiMAX complexes. First, we diluted 5 pmol of RNAi in 50 µl OptiMEM without serum, followed by the dilution of 1 µl of Lipofectamine RNAiMAX in 50 µl of OptiMEM. Finally, the diluted RNAi and the diluted Lipofectamine were combined and incubated for 20 min at room temperature, forming the RNAi-Lipofectamine RNAiMAX complexes. These complexes were added to each well containing cells and incubated 24-48 hours at 37°C in a CO2 incubator. For suspension

cells, we used the same protocol with a few changes. In this case, we used 6-well multiwell plates and the RNAi-Lipofectamine RNAiMAX complexes were formed with 30 pmol of RNAi in 150µl of OptiMEM.

3.6) RNA extraction and cDNA synthesis

Total RNA, including small RNA species, was extracted from U87CD133^{-}/U87 CD133^{+} cells using the miRCURY Isolation Kit – Cells (Exiqon), according to the recommendations of the manufacturer for cultured cells. Briefly, after cell lysis, the total RNA was adsorbed to a matrix, washed with the recommended buffers and eluted with 35 µL RNase-free water by centrifugation. After RNA quantification, cDNA conversion for miRNA quantification was performed using the Universal cDNA Synthesis Kit (Exiqon). For each sample, cDNA for miRNA detection was produced from 20 ng total RNA, according to the following protocol: 60 min at 42ºC followed by heat-inactivation of the reverse transcriptase for 5 min at 95ºC. The resulting cDNA was diluted 40 times with RNase-free water before quantification by qPCR.

Synthesis of cDNA for mRNA quantification was performed using the NZY First-Strand cDNA Synthesis Kit (NZYtech, Lisbon, Portugal) employing 1 µg total RNA for each reaction, by applying the following protocol: 10 min at 25ºC, 30 min at 50oC and 5 min at 85oC. After transcription, the samples were further incubated for 20 min at 37ºC with an RNase H (from *E. coli*) to specifically degrade the RNA template in cDNA:RNA hybrids after first-strand cDNA synthesis. Finally, the obtained cDNA was diluted 10 times with RNase-free water before quantification by qRT-PCR.

3.7) Quantitative Real-time PCR

Quantitative real time PCR was performed in a StepOnePlus thermocycler (Applied Biosystems) using 96-well microtitre plates.

For microRNA quantification the miRCURY LNATM Universal RT microRNA PCR system (Exiqon) was used in combination with pre-designed primers (Exiqon) for miR-128. The small nuclear RNA snord44 was used as reference. A master mix was prepared for each primer set, according to the recommendations for real-time PCR setup of

individual assays suggested in this kit. For each reaction, 6 μL of master mix was added to 4 μL template cDNA. All reactions were performed in duplicate (two cDNA reactions per RNA sample) at a final volume of 10 μL per well, using the StepOnePlus software (Applied Biosystems). The reaction conditions consisted of polymerase activation/denaturation and well factor determination at 95ºC for 10 min, followed by 45 amplification cycles at 95ºC for 10s and 65ºC for 1 min.

For mRNA quantification, the iQ SYBR Green Supermix Kit (Bio-Rad) was used. The primers for the target gene BMI and for the reference gene HPRT were pre-designed by Qiagen (QuantiTect Primer, Qiagen, Hilden, Germany). A master mix was prepared for each primer set, containing a fixed 6.5 μL volume of SYBR Green Supermix and the appropriate amount of each primer to yield a final concentration of 150 nM. For each reaction, 10 μL of master mix were added to 2.5 μL of template cDNA. All reactions were performed in duplicate (two cDNA reactions per RNA sample) at a final volume of 12.5 μL per well, using the StepOnePlus software (Applied Biosystems). The reaction conditions consisted of enzyme activation and well-factor determination at 95ºC for 1 min and 30 s, followed by 40 cycles at 95ºC for 10 s (denaturation), 30 s at 55ºC (annealing), and 30 s at 72ºC (elongation).

For both miRNA and mRNA quantification, a melting curve protocol was started immediately after amplification and consisted of 1 min heating at 55ºC followed by 80 steps of 10 s, with a 0.5ºC increase at each step. The miRNA and mRNA fold change with respect to control samples was determined by the Pfaffl method, taking into consideration the different amplification efficiencies of all genes and miRNAs analyzed in each experiment. The amplification efficiency for each target or reference RNA was determined according to the formula: $E = 10(-1/S) – 1$, where S is the slope of the obtained standard curve.

3.8) MiRNA PCR panel

MicroRNA quantification using the 96-well miRNA PCR plates (Exiqon) was performed in an iQ5 thermocycler using the SYBR® Green Master Mix (Exiqon). The primers for the target miRNAs are displayed in table 6.

Table 6 – Target sequence of miRNAs detected using the miRNA PCR plates.

microRNA Name	Target sequence
hsa-let-7b	UGAGGUAGUAGGUUGUGUGGUU
hsa-miR-101	UACAGUACUGUGAUAACUGAA
hsa-miR-106a	AAAAGUGCUUACAGUGCAGGUAG
hsa-miR-106b	UAAAGUGCUGACAGUGCAGAU
hsa-miR-10b	UACCCUGUAGAACCGAAUUUGUG
hsa-miR-124	UAAGGCACGCGGUGAAUGCC
hsa-miR-128	UCACAGUGAACCGGUCUCUUU
hsa-miR-130a	CAGUGCAAUGUUAAAAGGGCAU
hsa-miR-130b	CAGUGCAAUGAUGAAAGGGCAU
hsa-miR-132	UAACAGUCUACAGCCAUGGUCG
hsa-miR-135b	UAUGGCUUUUCAUUCCUAUGUGA
hsa-miR-148a	UCAGUGCACUACAGAACUUUGU
hsa-miR-149	UCUGGCUCCGUGUCUUCACUCCC
hsa-miR-17	CAAAGUGCUUACAGUGCAGGUAG
hsa-miR-181a	AACAUUCAACGCUGUCGGUGAGU
hsa-miR-181c	AACAUUCAACCUGUCGGUGAGU
hsa-miR-185	UGGAGAGAAAGGCAGUUCCUGA
hsa-miR-188-5p	CAUCCCUUGCAUGGUGGAGGG
hsa-miR-19b	UGUGCAAAUCCAUGCAAAACUGA
hsa-miR-123	UCCUUCUGCUCCGUCCCCCAG
hsa-miR-200c	UAAUACUGCCGGGUAAUGAUGGA
hsa-miR-203	GUGAAAUGUUUAGGACCACUAG
hsa-miR-20a	UAAAGUGCUUAUAGUGCAGGUAG
hsa-miR-21	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-210	CUGUGCGUGUGACAGCGGCUGA
hsa-miR-25	CAUUGCACUUGUCUCGGUCUGA
hsa-miR-26a	UUCAAGUAAUCCAGGAUAGGCU
hsa-miR-27a	UUCACAGUGGCUAAGUUCCGC
hsa-miR-29b	UAGCACCAUUUGAAAUCAGUGUU
hsa-miR-30a	UGUAAACAUCCUCGACUGGAAG
hsa-miR-30c	UGUAAACAUCCUACACUCUCAGC
hsa-miR-32	UAUUGCACAUUACUAAGUUGCA
hsa-miR-328	CUGGCCCUCUCUGCCCUUCCGU
hsa-miR-34a	UGGCAGUGUCUUAGCUGGUUGU
hsa-miR-367	AAUUGCACUUUAGCAAUGGUGA
hsa-miR-448	UUGCAUAUGUAGGAUGUCCCAU
hsa-miR-451	AAACCGUUACCAUUACUGAGUU
hsa-miR-566	GGGCGCCUGUGAUCCCAAC
hsa-miR-573	CUGAAGUGAUGUGUAACUGAUCAG
hsa-miR-623	AUCCCUUGCAGGGGCUGUUGGGU
hsa-miR-7	UGGAAGACUAGUGAUUUUGUUGU
hsa-miR-9	UCUUUGGUUAUCUAGCUGUAUGA
hsa-miR-92a	UAUUGCACUUGUCCCGGCCUGU
hsa-miR-93	CAAAGUGCUGUUCGUGCAGGUAG

A master mix was prepared for each sample, containing equal volumes (1:1) of SYBR Green master mix and diluted cDNA. For each reaction, performed in duplicate, 10 µl of master mix were added per well. Reaction conditions and melting curve protocol were similar to those described for qPCR quantification of miRNA expression. Threshold values for threshold cycle determination (Ct) were generated automatically by the iQ5 Optical System Software. Relative miRNA level calculation and statistical analysis were performed using the software qBasePlus software (Biogazelle, Gent, Belgium).

3.9) Assessment of Nestin and CD133 expression by Flow Cytometry

To evaluate the expression of nestin and CD133, U87 cells bounded (U87/CD133^{+}) an unbounded (U87/CD133^{-}) to CD133 microbeads, cells were plated into 6-well plates in the conditions referred in section 3.3). Since U87/CD133^{-} grow in adherent conditions, in the day of flow cytometry experiments these cells were washed twice with PBS, detached from plates by exposure to dissociation medium (5 min, 37ºC) and washed once more with PBS. Both cell types (U87/CD133^{-} and U87/CD133^{+}) were then ressuspended in 500 µL of cold PBS. After washing, cells were incubated with an antibody for CD133/nestin (1:500) for 30 minutes. Since nestin is an intracellular protein, before incubation with the antibody against nestin cells were permeabilized with a solution containing (PBS 1x, 0,1% triton and 2% FBS). After incubation with the antibodies, cells were washed one more time with 500 µL of PBS and finally incubated with alexa-488 secondary antibody (1:200), if necessary. After a final washing step, cells were analyzed in a FACS Calibur flow cytometer (BD, Biosciences). Alexa-488 fluorescence was evaluated in the FL-1 channel and a total of 10.000 events were collected for each sample. All data were analyzed using the Cell Quest software (BD).

3.10) Laminin coating

In order to test the behavior of GSC in the presence of laminin, we used laminin coated tissue culture plastic (Sigma: L2020). The working laminin solution (10ug/ml in PBS) was prepared freshly for each experiment by diluting the stock solution (1mg/ml) 1:100. Plates and flasks were covered with the diluted solution and incubated at 37ºC for at least 3 h.

3.11) Preparation of targeted SNALPS and evaluation of cellular assosiation

Briefly, CTX was modified by the addition of thiol groups upon reaction with freshly prepared 2-iminothiolane hydrochloride (2-IT, in HEPES-buffered saline pH 8) at a molar ratio of 1:10 (CTX: 2-IT). The reaction occurred under gentle stirring for 1 hour in the dark, at room temperature (RT). Thiolated CTX was then coupled to DSPE-PEG-MAL micelles, prepared in MES buffer pH 6.5,15 by a thioesther linkage (1:1, CTX: DSPE-PEG-MAL molar ratio). The coupling reaction was performed overnight (at RT) in the dark with gentle stirring. For the NT SNALPs, post insertion was performed with plain micelles (without conjugated ligand), which were prepared by adding HEPES-buffered saline (pH 8.0) to the DSPE-PEG-MAL micelles. The neutralization of free maleimide groups in the micelles was carried out upon incubation with β-mercaptoethanol at a maleimide: β-mercaptoethanol molar ratio of 1:5 (0.52:2.6 μmol), under stirring for 30 minutes (at RT). The insertion of CTX-DSPEPEG-MAL conjugates or plain DSPE-PEG-MAL micelles onto the preformed liposomes, at 4 mol% (relative to the total lipid concentration), was performed upon incubation in a water bath at 39 °C for 16 hours (in the dark). Targeted and NT SNALPs were purified by size exclusion chromatography on a Sepharose CL-4B column using HEPES-buffered saline (pH 7.4) as running buffer to remove non-conjugated micelles and chemical reagents (including unreacted 2-IT and β-mercaptoethanol) used during SNALPs preparation. To evaluate the extent of cellular association of the SNALPs, cells were plated onto 48-well plates at densities of $5 \times 10^{4.}$ Twenty-four hours after plating, cells were incubated in OptiMEM (Gibco) with targeted CTX-coupled or NT liposomes encapsulating FAM-labeled oligonucleotides for 4 hours at 37 °C. Subsequently, cells were washed twice with cold PBS (pH 7.4), detached by exposure to trypsin (5 minutes, 37 °C) and further washed twice with PBS. Cells were then ressuspended in 350 μl of cold PBS and immediately analyzed in a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). FAM fluorescence was evaluated in the FL-2 channel and a total of 20,000 events were collected for each sample (unless stated otherwise). The data were analyzed by Cell Quest software (BD Biosciences). Trypan blue was added (10μL) to quench the fluorescence in the extracellular medium

Chapter 4 Results

4.1) U87-derived cancer stem cells form neurospheres when cultured under non-adherent conditions

Recently, cancer stem cells (CSCs) have emerged as a focus of debate in the development of new therapeutic strategies. It seems essential to find differences between CSCs and differentiated cancer cells in order to understand why CSCs are more resistant to therapies, with the ultimate goal of creating specific treatments that target these cells and improve glioblastoma patient survival. In this study we proposed to isolate glioblastoma stem cells from a human GBM cell line (U87), using CD133 as a marker, and culture these cells in the form of neurospheres.

4.1.1) Isolation of CSCs from U87 cells using the magnetic associated cell sorting system

Our first goal in this project was to isolate CSCs from U87 cells, a well-known human GBM cell line. For this purpose, we used magnetic associated cell sorting (MACS) and selected CD133 as the specific cell marker to identify the CSC population. During the sorting process, U87 cells were incubated with magnetic microbeads that specifically bind to epitope 1 of the human CD133 antigen. By applying a magnetic field, it was possible to retain the cell population that was bound to the magnetic beads in a column, resulting in the separation of these cells from the unbound cells. One portion of bound cells was cultured in DMEM/F12 (Invitrogen) supplemented with 1% N2 and 2% B27 (Invitrogen) and 20 ng/mL epidermal growth factor and fibroblast growth factor.

Initially, in order to evaluate the percentage of bound cells that were positive for CD133, a small sample of bound-cells was incubated with an antibody associated with a fluorophore (PE) against the epitope 2 of the human CD133 antigen. However, as illustrated in Figure 9, no significant difference in FL-2 fluorescence was observed between cells incubated with the isotype antibody and cells incubated with the anti-CD133 antibody. To ensure that the presence of the magnetic microbeads was not preventing antibody binding to CD133, we repeated the experiment two weeks after cell isolation. However, once again, the results showed a lack of labeling for bound cells in the presence of the anti-CD133 antibody (data not shown).

In face of these negative results, we examined whether the chosen antibody was working properly. , by employing HT-29 cells, a human colon tumor cell line known to express

CD133 as a positive control for CD133 labeling. Our results, illustrated in Figure 10, suggested that the anti-CD133 antibody was not working properly, since no labelling was observed in this cell line.

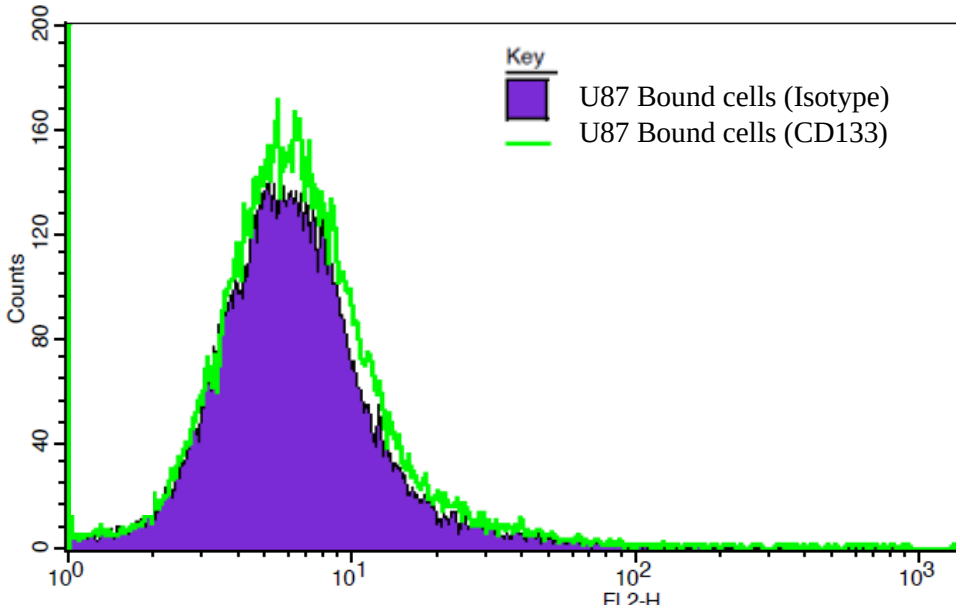


Figure 9 – Expression of CD133 marker in U87 Cells bound to microbeads. Cells were incubated with an antibody associated with the fluorophore PE that recognize epitope 2 of the human CD133 antigen. The percentage of cells expressing CD133 was assessed by flow cytometry (Purple – fluorescence of isotype in cells bound to microbeads and Green - fluorescence of CD133 in cells bound to microbeads)

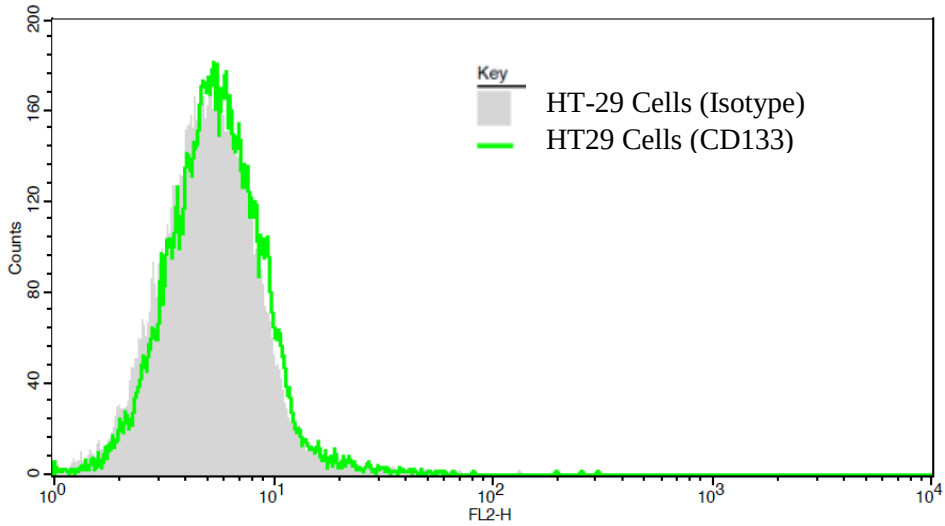


Figure 10 -– Expression of CD133 marker in HT-29 Cells. Cells were incubated with an antibody associated with a fluorophore (PE) that recognized epitope 2 of the human CD133 antigen. The percentage of cells expressing CD133 was assessed by flow cytometry (Grey – fluorescence of isotype in HT-29 cells. Green – fluorescence of CD133 in HT-29 cells.

After acquiring a new antibody against CD133, our first step was to ensure that this antibody was working properly. For this purpose,we incubated HT-29 cells with the new CD133 antibody and a secondary Alexa-488 antibody. The number of CD133 positive cells was once again assessed by flow cytometry and, as observed in Figure 11), we were able to observe that 70% of the cell population expressed CD133.

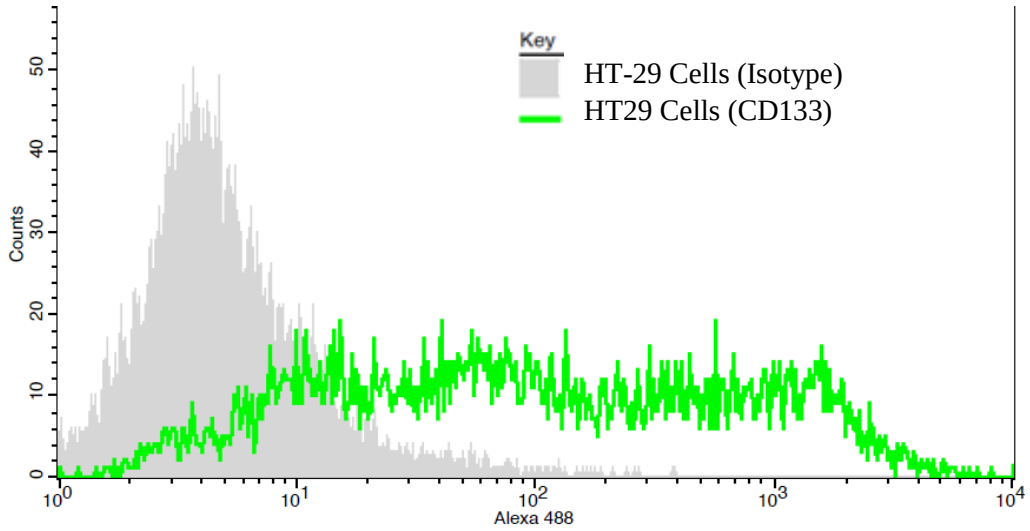


Figure 11 - Expression of CD133 marker plus Alexa-488 in HT-29 Cells. Cells were incubated with a primary antibody associated with a fluorophore (PE) that recognize epitope 2 of the human CD133 antigen and with a secondary Alexa-488 antibody. The percentage of cells expressing CD133 was assessed by flow cytometry (Grey – fluorescence of isotype in HT-29 cells. Green - fluorescence of CD133 in HT-29 cells).

We then proceeded to the incubation of U87 cells bound to magnetic microbeads with the new antibody. The percentage of cells expressing the CD133 marker was assessed by flow cytometry based on the Alexa-488 fluorescence (Figure 12). Cells incubated only with the secondary antibody Alexa- 488 were used as a control. Figures 12a and 12c show that an average of 40% of the cells bound to microbeads express the CD133 marker. Results from experiments in which the unbound cells ($CD133^-$) were subjected to the same procedure (Figure 12b) showed that only 8% of this population expressed the CD133 marker (Figure 12c).

To further validate our results in what concerned the cancer stem cell nature of the bound cells, we incubated bound and unbound cells with an antibody against nestin, another CSC marker, and with an Alexa-488 secondary antibody and the percentage of $nestin^+$ cells in each population (bound and unbound cells) was assessed by flow cytometry (Figure 13). $CD133^+/CD133^-$ cells incubated with the secondary antibody Alexa-488 were used as a control. The population of bound cells showed an average of 75% $nestin^+$

cells (Figure 13a and 13c). As described previously, we also quantified nestin expression in CD133$^-$ cells to evaluate whether all stem cell-like GBM cells have been isolated through the MACS procedure (Figure 13b). The results showed that CD133$^-$ cells have an average of 30% nestin$^+$ cells (Figure 13c).

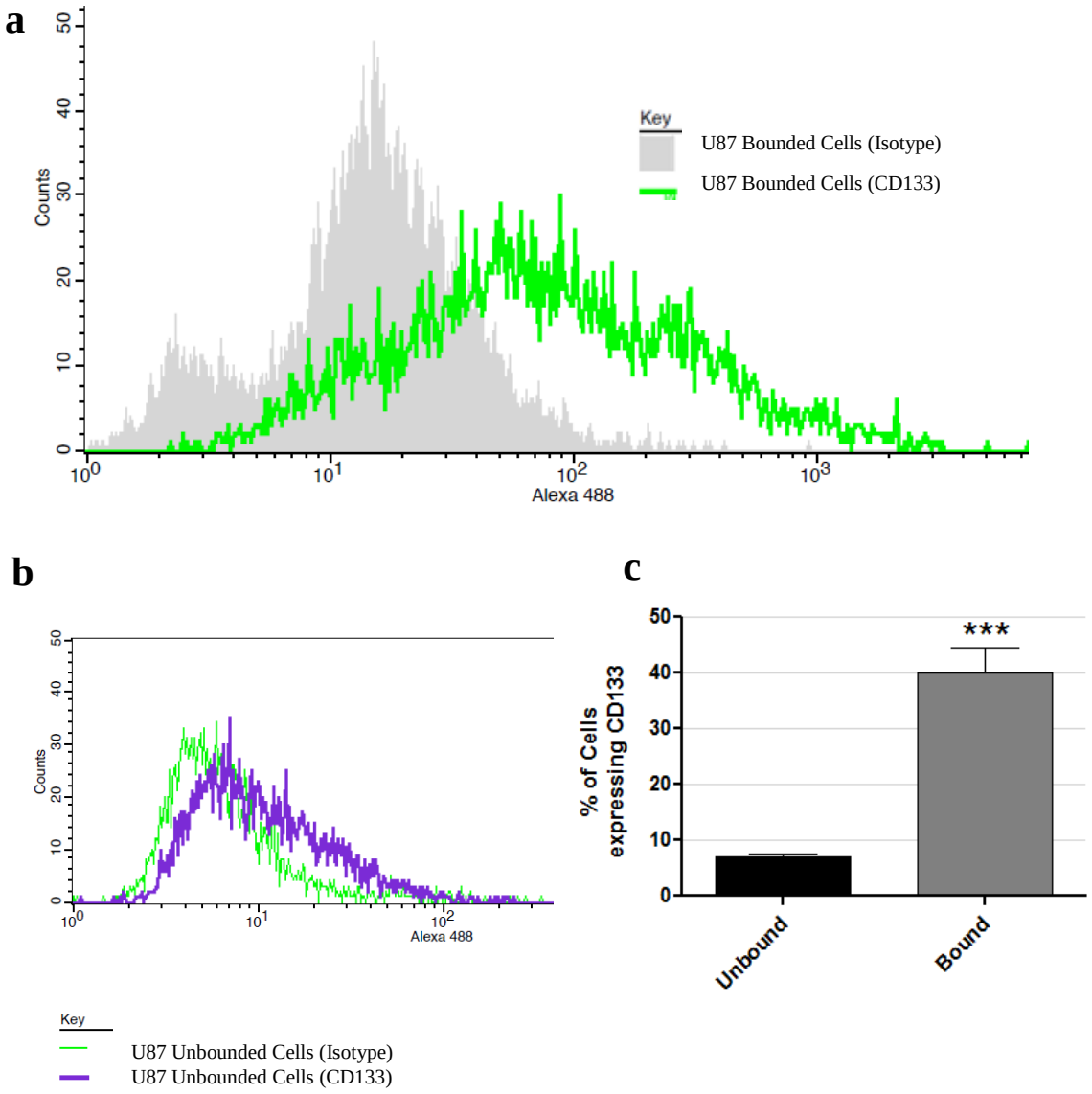


Figure 12 – Expression of CD133 marker in U87 bound and unbound cells. Bound and unbound cells were incubated with CD133 antibody followed by incubation with an alexa-488 anti-mouse secondary antibody**. a)** Flow cytometry histogram showing the expression of CD133 in cells bounded to microbeads and cultured in DMEMF12 in non-adherent conditions. (Green – fluorescence of CD133 in cells bounded to microbeads and Grey - fluorescence of isotype in cells bounded to microbeads)**. b)** Flow cytometry histogram showing the expression of CD133 in cells unbounded to microbeads and cultured in DMEM in adherent conditions (Purple – fluorescence of CD133 in cells unbounded to microbeads and Green - fluorescence of isotype in cells unbounded to microbeads)**. c)** Percentage of CD133$^+$ cells (Bounden and unbounded cell populations). The results are presented as the percentage of CD133+ cells with respect to the control (cells incubated with the secondary antibody alexa-488). The results are representative of three independent experiments. * – P < 0.05, ** – P < 0.01, *** – P < 0.001

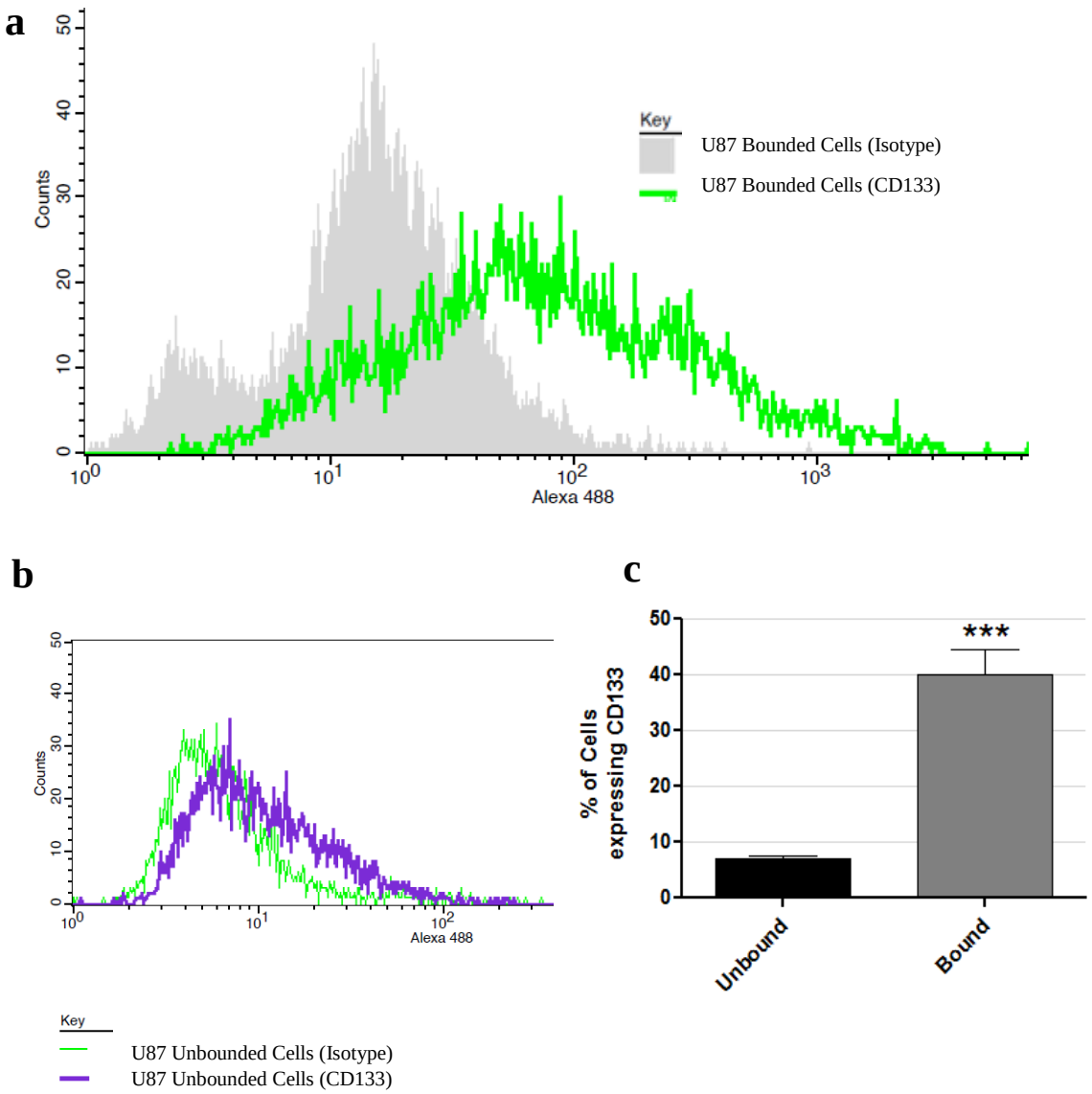


Figure 13 – Expression of nestin in bound and unbound cells. Bound and unbound cells were incubated with an anti-nestin antibody followed by incubation with the alexa-488 secondary antibody. **a**) Flow cytometry histogram showing the expression of nestin in bound cells cultured in DMEMF12 in non-adherent conditions. **b)** Flow cytometry histogram showing the expression of nestin in unbound cells cultured in DMEM in adherent conditions. **c)** Percentage of nestin^{+} cells (Bound and unbound cell populations). The results are presented as the percentage of CD133+ cells with respect to the control (cells incubated with the secondary antibody Alexa-488). The results are representative of three independent experiments. * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$.

4.1.2) Neurosphere formation by CD133$^+$ cells in DMEMF12 medium

Our second goal was to and maintain the cancer stem cell properties of CD133$^+$ cells during the subsequent experiments. For this purpose, after the isolation of CD133$^+$ cells, these cells were cultured in non-adherent conditions, in DMEM/F12 medium supplemented with 1% N2 and 2% B27 (Invitrogen) and 20 ng/mL epidermal growth factor and fibroblast growth factor. When cultured under these conditions, CD133$^+$ cells formed 3-D clusters, called neurospheres.

Neurospheres were formed over period of two weeks and presented different diameters (Figure 14a). No neurosphere formation was observed when CD133$^-$ cells were cultured in similar conditions (Figure 14b). As shown in Figure 14c and 14d, when cultured in adherent conditions with DMEM, both CD133$^-$ and CD133$^+$ cells failed to form neurospheres, growing at a similar rate.

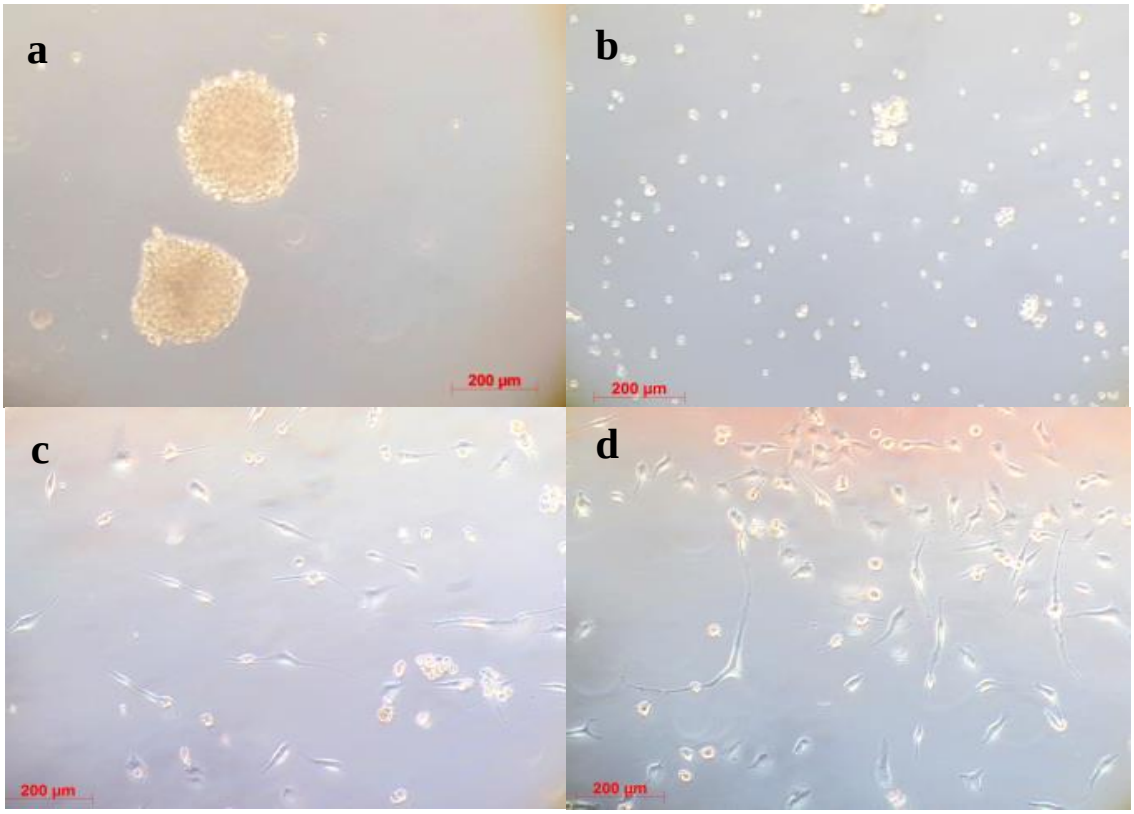


Figure 14 – Representation of U87/ (CD133$^+$ /CD133$^-$) cells cultured in different conditions. a) CD133$^+$ and **b)** CD133$^-$ cells were cultured in DMEM/F12 medium supplemented with 2% B27 and 20 ng/mL epidermal growth factor and fibroblast growth factor in low-adherence wells. Neurospheres were formed in U87/CD133$^+$ cells two weeks after isolation from the U87 cell line. **c)** CD133$^+$ and **d)** CD133$^-$ cells were cultured in adherent conditions with DMEM. No neurospheres were formed in both cell populations in these conditions.

In conclusion, $CD133^+$ cells isolated from the human glioma cell line U87 present two of the major hallmarks of glioblastoma stem cells, which are the surface expression of cancer stem cell markers (nestin and CD133) and the ability to grow as neurospheres.

4.2) Glioma stem cells show different miRNA profiles when compared to differentiated glioma cells.

MicroRNAs regulate many important processes, such as neuronal differentiation, cell growth, proliferation and apoptosis. For this reason, we believe that these small RNA molecules can be responsible for the unique characteristics of CSCs. Recent studies have shown that miRNAs are important for the high resistance and self-renewal of CSCs. To further clarify this assumption, we decided to compare the miRNA profile of glioblastoma stem cells (GSCs) with that of non-stem glioblastoma cells, using pre-designed qPCR plaques containing primers for 44 miRNAs involved in the cancer biology.

Using miRNA qRT-PCR arrays, we identified several miRNAs deregulated in glioma stem cells ($CD133^+$) with respect to differentiated glioma cells ($CD133^-$). As shown in Figure 15, several miRNAs have their expression modified in GSCs, with respect to the remaining glioblastoma cell population.

MicroRNA-128, a well-known miRNA described to be downregulated in glioblastoma, was shown to have a very low expression in GSCs. From all tested miRNAs, this was the one presenting the largest difference in expression levels between the $CD133^+$ and $CD133^-$ population. Several other miRNAs had their expression slightly downregulated in GSCs, such as miR-130a, miR-1237, miR-210, miR-92a, miR-10b and miR-124

On the other hand, several miRNAs were shown to be upregulated in GSCs with respect to the remaining glioma cell population. The most upregulated miRNAs found in this experiment were miR-25, miR-29b, miR-26a, miR-328, miR-101, miR-181a, miR-21, miR-27a, miR-25, miR-30a, miR-30c and miR-32. Several of these miRNAs have been widely studied in the context of glioblastoma, such as miR-21 and miR-181a, and have important roles in tumor growth and cell proliferation. Several other miRNAs presented a slightly upregulated expression, including let-7b, miR-130a, miR-149, miR-19b, miR-34a, miR-9, miR-17, miR-106a, miR-130b, miR-185, miR-20a and miR-93.

For the other studied miRNAs no difference in their expression levels between GSCs and the remaining glioblastoma cell population were observed (data not shown)

In conclusion, GSCs and the remaining glioblastoma cell population showed different miRNA profiles. Among the deregulated miRNAs, miR-128 presented the most altered expression, being highly downregulated in GSCs.

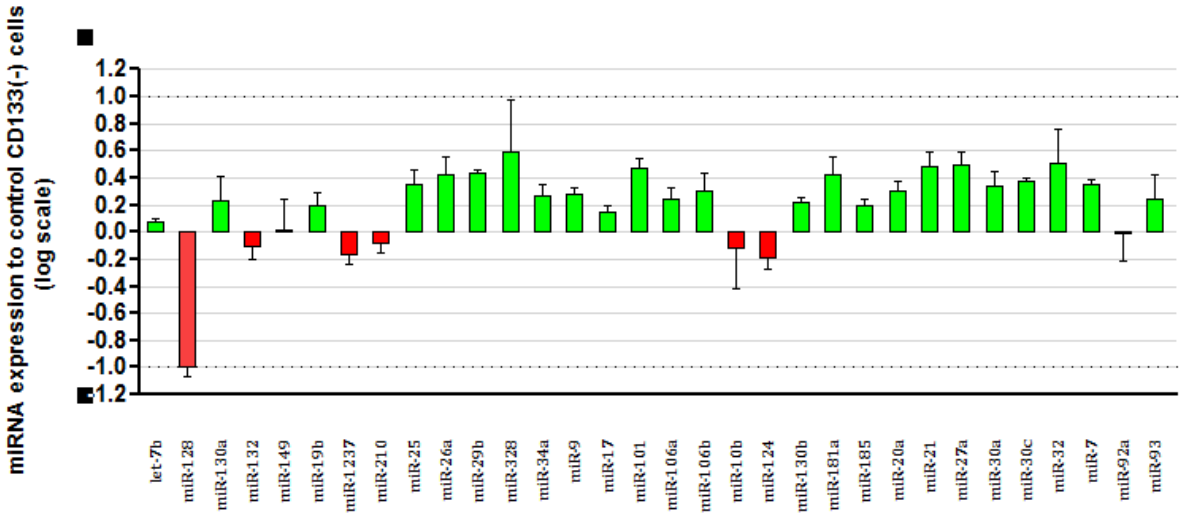


Figure 15 – MiRNAs expression comparison between GSCs and differentiated glioblastoma cells. QPCR quantification of 44 miRNAs in GSCs ($CD133^+$) and glioblastoma cells ($CD133^-$) cells was performed using pre-designed miRNA PCR plates. Ct values were obtained for each sample (threshold=40 cycles) and normalized to reference gene - snord44; Relative miRNA expression values were calculated using the qBasePlus software. MicroRNAs not showed either had no different levels of expression between $CD133^-$ and $CD133^+$ cells or were not detected by qPCR. The results are representative of three independent experiments.

4.3) MicroRNA-128 sensitizes U87 to sunitinib-induced cell death

In the previous section, using pre-designed qPCR plates we were able to determine different patterns of miRNA expression between GSCs ($CD133^+$) and the remaining glioblastoma cells ($CD133^-$). These results, together with the fact that miRNAs have been linked to many disease processes involving stem cells are strong indications that miRNAs are important for the unique biology of GSCs.

Our next goal was to prove that reverting the expression patterns of these miRNAs could impair normal GCS function and, consequently, glioblastoma cell growth, setting the basis for new therapeutic strategies against this type of cancer.

Since our results showed that miR-128 exhibited the most altered expression between GSCs ($CD133^+$) and the remaining glioblastoma cell population ($CD133^-$), we decided to study this miRNA and its targets in more detail. For this purpose, we transfected the whole U87 cell population (adherent conditions in DMEM medium) and U87/CD133+ cells (neurospheres in DMEM/F12 medium) with miR-128 mimics using Lipofectamine RNAiMAX. Lipofectamine RNAiMAXis a commercially available and efficient reagent for RNAi delivery to a wide variety of cell lines, stem cells and primary cells. As a control, in this experiment, we used non-transfected cells and cells transfected with a scrambled mimic (control mimic).

As shown in Figure 16, miR-128 intracellular levels were successfully increased, in U87 cells, as assessed by qRT-PCR. Unfortunately, no increase in miR-128 levels were observed in neurospheres originated from U87/ $CD133^+$ cultures (data not shown).

According to the literature, miR-128 has several validated targets (Table 7). Among them, BMI-1 (Figure 17b) is one of the most studied and has been linked to glioma stem cell resistance to therapy[105]. To evaluate if miR-128 increase led to a downregulation of BMI-1 in U87 cells, we performed qRT-PCR experiments and as illustrated in figure 17c BMI-1 levels are significantly decreased in the U87 human cell line, as compared to controls.

Taking these results into consideration, we started a series of experiments employing sunitinib, in order to evaluate if the cytotoxic effect of this tyrosine kinase inhibitor could be potentiated and therefore reduce its therapeutic dose upon combination of this drug with miR-128 mimics. Figure 18 shows that miR-128 mimics or sunitinib (15µM) alone did not decrease cell viability. However, when combined, miR-128 and sunitinib were

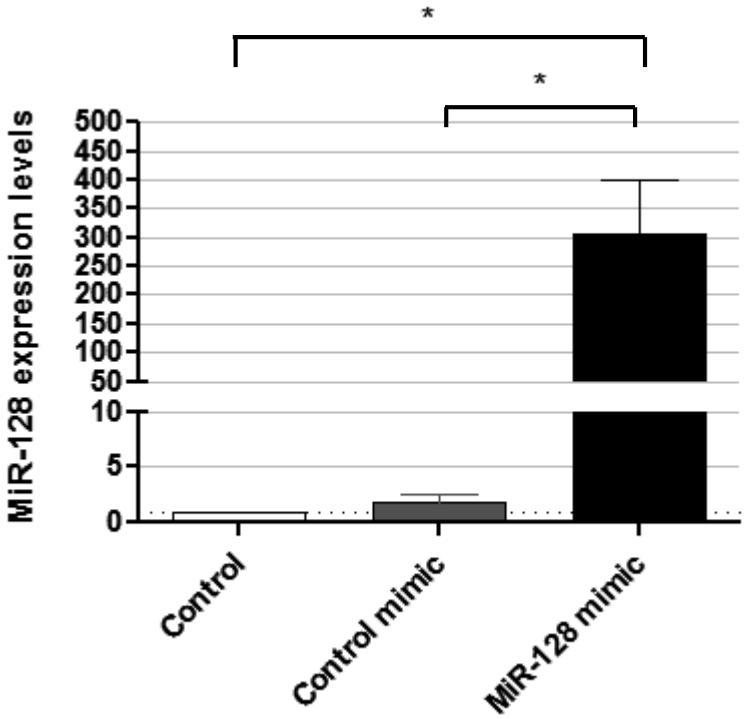


Figure 16 - Evaluation of miR-128 expression levels in U87 cells following transfection with miR-128 mimics. Cells were transfected with miR-128 mimics or control mimics using Lipofectamine RNAiMAx for 48 hours. miR-128 levels were quantified by qRT-PCR in a StepOnePlus thermocycler (Applied Biosystems) using 96-well microtitre plates and were normalized using SNORD 44 as the reference gene.

able to reduce cell viability to approximately 20%, a result similar to what can be achieved with a higher concentration (30 μM) of the drug.

To overcome the limitation associated with the difficulty of transfecting U87/CD133+ cells, we developed two possible strategies to improve transfection. The first strategy was based on the use of laminin-coated plates, while the second strategy focused on the use of chlorotoxin-coupled stable nucleic acid lipid particles (SNALPs).

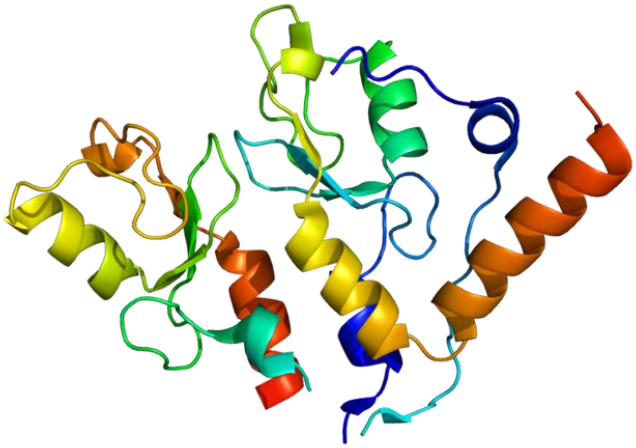
Laminin-coated plates are a new approach to study cancer stem cells. This culture method allows cancer stem cells to grow adherent to a surface without losing their stem properties. In this regard, laminin plates were prepared by adding laminin to the wells and incubating plates at 37ºC for at least for 3 hours. In order to verify if U87/CD133^{+} cells cultured in

laminin-coated plates maintained their stem potential, we assessed the expression of the

a) **Table 7 – miR-128 Validated targets**

MicroRNA-128 Targets
BMI-1[105]
E2F3a[89]
PRC[90]
NTRK3[109]
ABCC5[110]

b)



c)

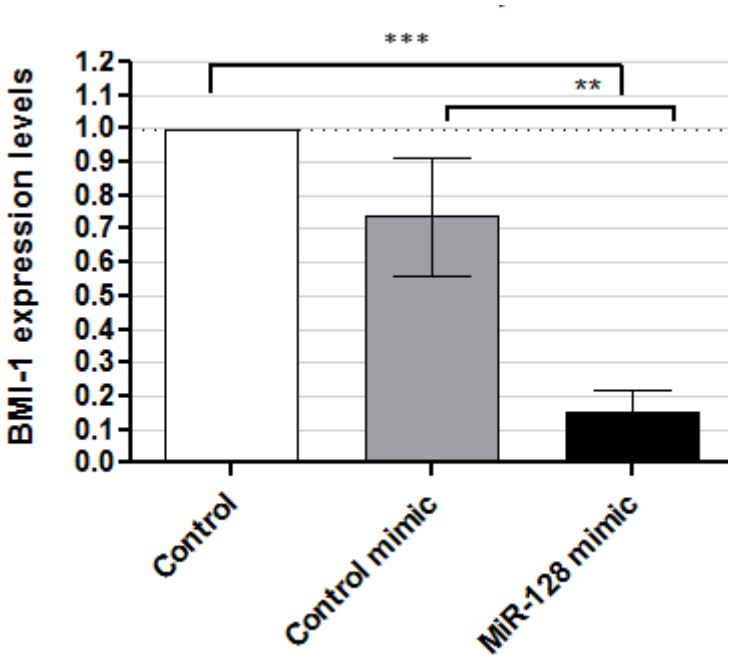


Figure 17 - Representation of miR-128 targets and BMI-1 expression levels following U87 transfection with miR-128 mimics. a) MicroRNA-128 validated targets b) PBD representation of BMI-1 protein. c) BMI-1 mRNA expression levels in U87 cell line. Cells were transfected with miR-128 mimic using Lipofectamine RNAiMAx and incubated for 48 hours.BMI-1 mRNA levels were quantified by qPCR in StepOnePlus thermocycler (Applied Biosystems) using 96-well microtitre plates and normalized using HPRT as the reference gene. Results are representative of three independent experiments. * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$

CD133 marker after 2 weeks, by flow cytometry, following cell incubation with CD133 antibody plus the secondary antibody Alexa-488. Figure 19 illustrates the obtained results and shows that 30% of cells cultured in laminin expressed CD133.

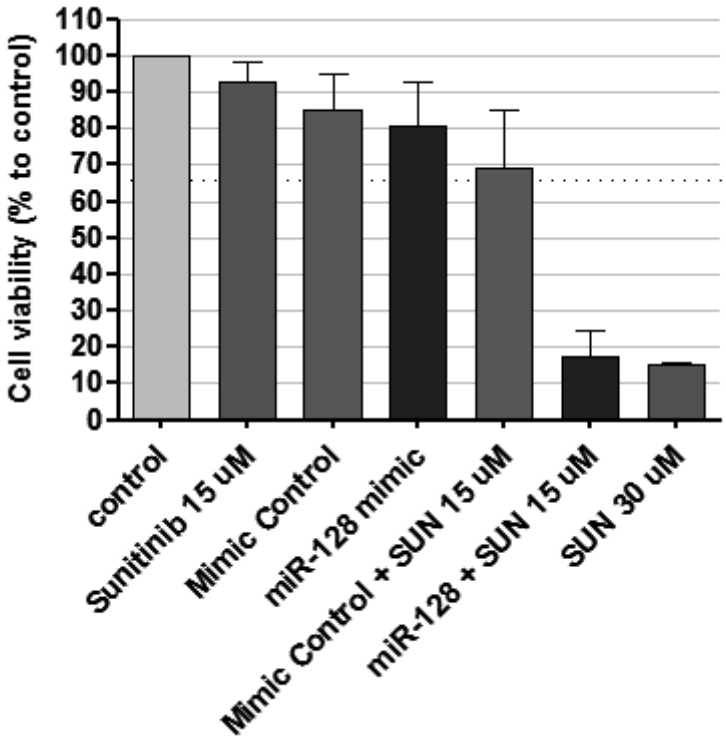


Figure 18 – U87 cell viability 48h hours after transfection with miR-128 mimics and/or exposure to sunitinib. Cells were transfected with miR-128 mimics using Lipofectamine RNAiMAx and incubated for 48 hours. After this period sunitinib was added to the medium and cells were further incubated for 24 hours. Cell viability was measured by the alamar blue assay 72 hours after transfection. Results were obtained from six independent experiments and were normalized to control (non-transfected cells) values. *p<0.05; **p<0.01; ***p <0.001.

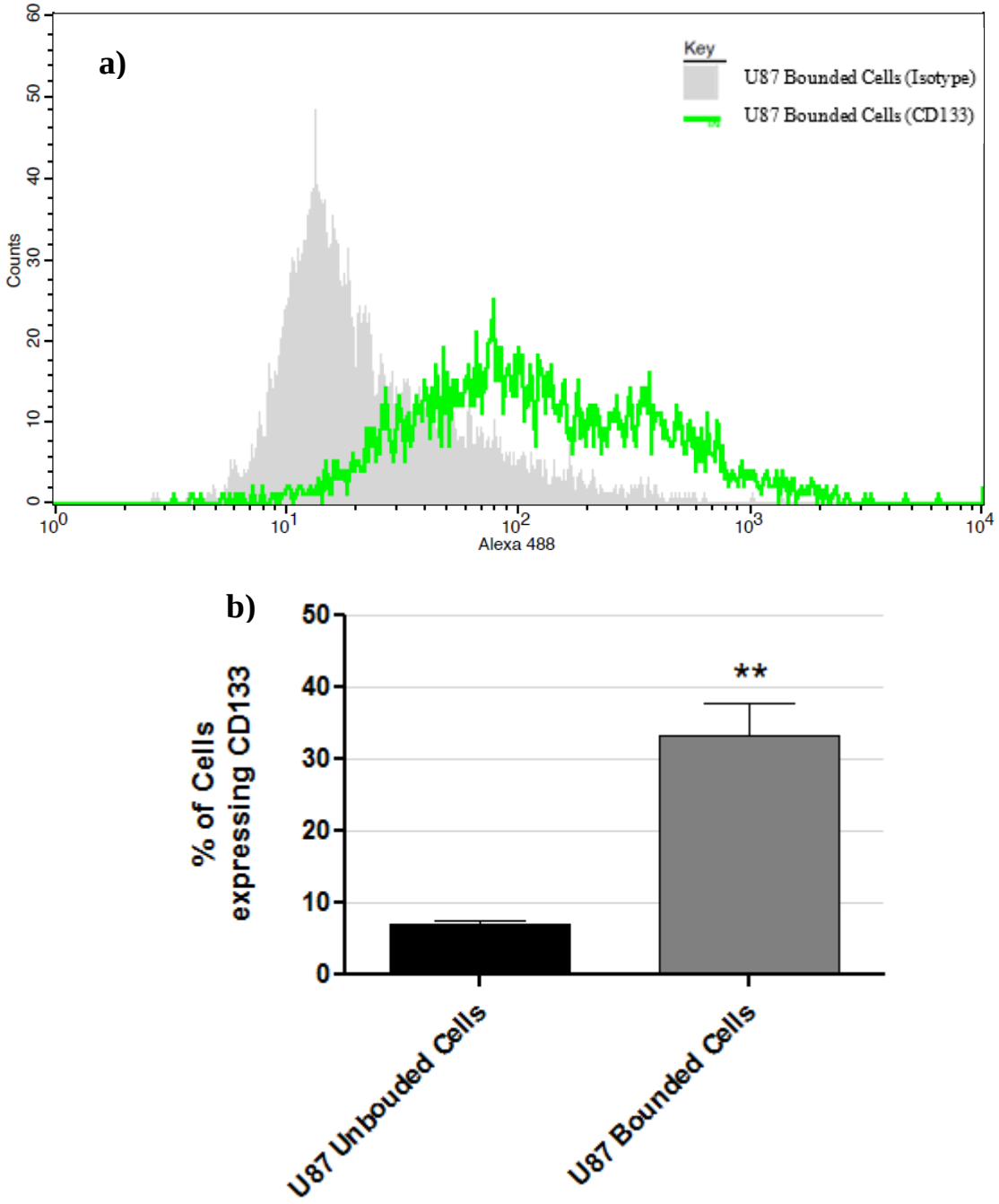


Figure 19 - Expression of CD133 marker in U87/CD133$^+$ cells cultured in laminin-coated plates. Following 10 days in culture in laminin-coated plates, CD133$^+$ cells were incubated with an antibody against CD133 and with a secondary antibody with alexa-488 associated. The percentage of cells expressing CD133 was assessed by flow cytometry. **a)** Flow cytometry histogram showing the expression of CD133 in cells bounded to microbeads, cultured in DMEM/F12 in laminin coated plates. Grey – expression of Isotype in cells bounded to microbeads and Green - expression of CD133 in cells bounded to microbeads) **b)** Percentage of cells expressing CD133 (Bounded and unbounded to microbeads). Both results were normalized with the control (isotope), which corresponds to cells incubated only with the secondary antibody alexa-488. The results are representative of independent experiments. * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$.

Another strategy explored in this work to improve the transfection efficiency of GSCs involved the use of targeted nanoparticles. Chlorotoxin-coupled stable nucleic acid lipid particles (SNALPs) were tested in 2013 in our lab, showing very promising results in what concerns the delivery of small interfering RNAs and anti-miRNA oligonucleotides to glioma cells[111]. Chlorotoxin (CTX) was modified by the addition of thiol groups, and thiolated CTX was then coupled to DSPE-PEG-MAL micelles through a thioesther linkage. U87/CD133^{+} cells were incubated with chlorotoxin (CTX)-coupled or nontargeted (NT) liposomes encapsulating FAM-labeled oligonucleotides, and the internalization of these nanoparticles was assessed by flow cytometry (Figure 20). To ensure that the detected fluorescence signal was due to the internalized SNALPs trypan blue was added to quench the fluorescence in the extracellular medium. Figure 20b shows that almost 90% of the cells internalized CTX-SNALPS. On the other hand, only 35% of the cells internalized NT-SNALPS.

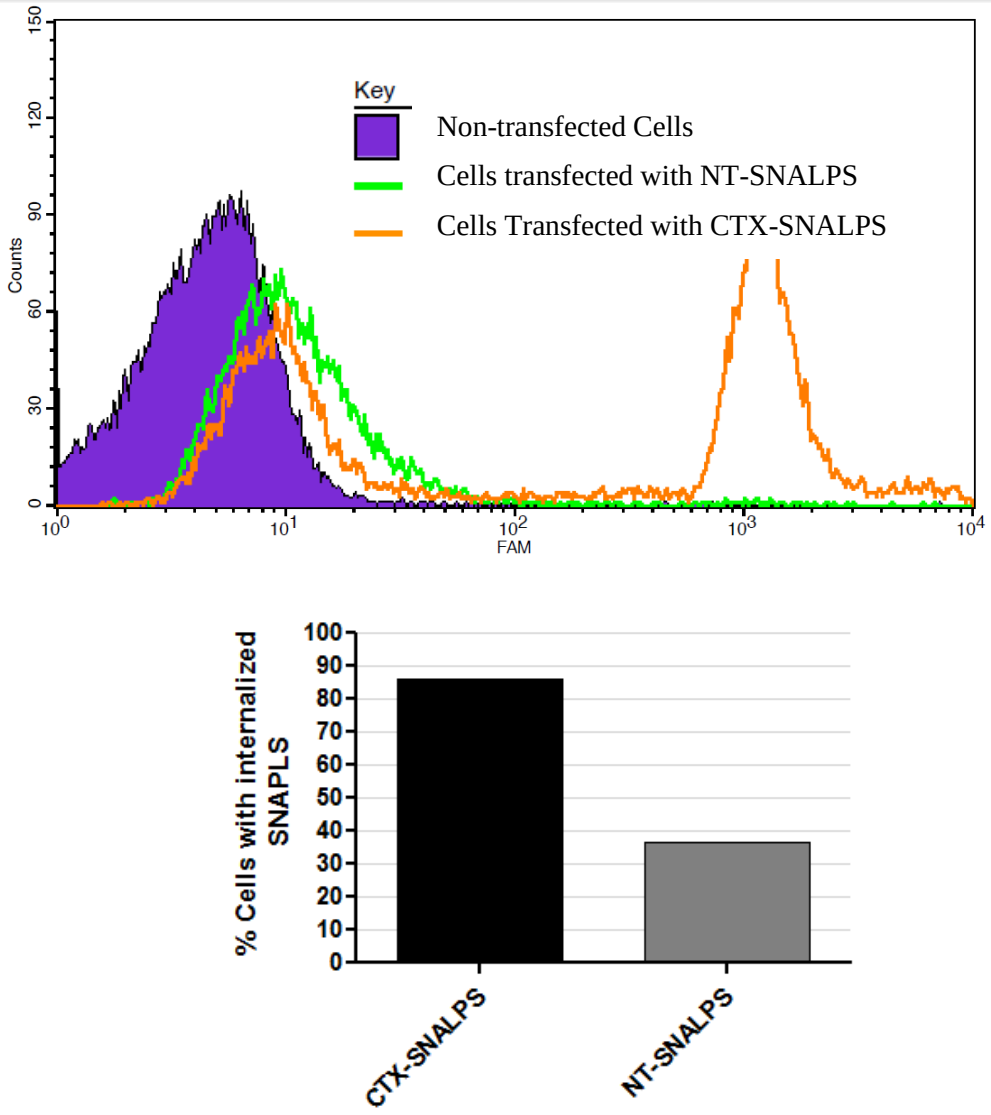


Figure 20 – Internalization of SNALPs in U87/CD133$^+$ cells cultured in laminin-coated plates. U87/CD133$^+$ cells were incubated with chlorotoxin (CTX)-coupled or nontargeted (NT) liposomes encapsulating FAM-labeled oligonucleotides. Particle internalization was assessed by flow cytometry. **a)** Flow cytometry histogram showing the internalization of green – NT-SNALPS and orange – CTX-SNALPS. **b)** Percentage of cells presenting internalized NT-SNALPS or CTX-SNALPS. The results are representative of one experiment,

Chapter 5 Discussion

5) Discussion

MicroRNAs have been associated with various important biological processes over the last decade. Regarding glioblastoma, there have been accumulated evidences of miRNA importance for cell proliferation, invasion and stem cell renewal. Several studies have reported miRNAs to be involved in GBM pathology, affecting multiple processes, including proliferation, invasion, migration, angiogenesis, resistance to therapy and apoptosis. These small RNA molecules have specific characteristics that make them desirable therapeutic targets, including their small size, tissue specificity and multi-targeting potential. That said, it seems obvious that these RNA molecules can be used as both therapeutic agents and therapeutic targets. However, for this to become a reality it is necessary to clarify the role of each miRNA in the biology of glioblastoma.

Another field of interest in glioblastoma research concerns cancer stem cells. Recent findings reported the existence cells with stem-like properties among the tumor cell population. These cells confer the tumor self-renewable and tumorigenic abilities and contribute to tumor resistance. In the last decade, cancer stem cells have also been identified in human glioma. However, in glioma, as well as in other cancer types, their role is not yet fully understood. It is common knowledge that these cells are able to generate the different type of cells that comprise the tumor, sustaining tumorigenesis. According to recent studies, GSCs are also more resistant to radio and chemotherapy. Taking into consideration their potential to form all kinds of tumor cells, GSCs may be responsible for the reappearance of the tumor even after its surgical removal. Therefore, therapies that directly target GSCs are essential for the complete eradication of this type of cancer.

As previously stated, miRNAs can control the translation of most protein-coding genes, and are involved in almost every biological pathway, including those connected with GSC biology. Over the past decade, numerous studies have helped to clarify the role of miRNA in CSCs biology. Nevertheless, further studies are required, including those concerning the comparison between miRNA profiles of GSC and the remaining glioblastoma cells. These studies can provide important clues to explain why GSC have unique properties, such as their high resistant to therapies. Also, taking into account the differences in the miRNA profile of GSCs, it would be possible to develop therapies specifically targeting these cells, thus expanding and optimizing the therapeutic options for glioblastoma.

In the present study, we aimed to compare miRNA profiles of glioma stem cells and differentiated glioma cells in order to identify alterations that could explain the different characteristics of both types of cells. By performing qRT-PCR arrays against 44 selected miRNAs, we showed that GSCs and the remaining glioblastoma cells have different miRNA profiles. We obtained evidences that miR-128, in particular, is highly downregulated in GSC. Furthermore, we observed that miR-128 overexpression sensitized U87 GBM cells to sunitinib-induced cellular death.

Initially, we isolated GSC from an established glioblastoma cell line (U87 cells) employing magnetic associated cell sorting, using CD133, a well-known cancer stem cell marker, as a marker for GSCs. We also employed a thoroughly validated protocol for GSC growth, using serum-free media supplemented with fibroblast growth factor and epidermal growth factor, in order to allow the formation of neurospheres, since the ability to form these structures is a major hallmark of GSCs. These conditions greatly reduce differentiation and are known to preserve genetic profiles similar to those found in tumors removed from patients with an enhanced GSC population. The absence of serum is essential since, accordingly with Singh et al[112], when exposed to serum, neurospheres start to differentiate down the lineage of the parent tumor.

Originally, the cells isolated with the CD133 microbeads, although forming neurospheres in culture, did not show CD133 labelling when tested with flow cytometry and an antibody against the marker (figure 9). We hypothesized that, despite the fact that our microbeads and CD133 antibody targeted different epitopes of the CD133 protein, the microbeads could cause a modification of the conformation of CD133 or even a stearic block effect that prevented the binding of the antibody. In order to investigate these possibilities, and the suggestion of the manufacturer, we incubate cells bounded to microbeads with CD133 antibody (PE) two weeks of the isolation. This waiting time was though to allow microbeads detachment from the cells. However, our results showed again no CD133 labeling. Taking these new results into consideration, we decided to test the antibody in the HT-29 cell line, which is known to express CD133. Since no CD133 labeling was also observed in this cell line (figure 10), we concluded that our antibody was not working properly and decided to acquire a similar antibody from a different brand.

Flow cytometry experiments employing the new antibody revealed that at least 37% of the microbead-bound cell population was $CD133^+$ (figure 12) and 77% of these population was also $nestin^+$ (figure 13). These results, together with the ability to form neurospheres (figure 14) allowed us to conclude that the microbead-bounded cell population had GSC properties.

Nevertheless, expression levels of CD133 were not very high (around 37 %), especially when compared with the results obtained by Christoph P. Beier and colleagues[62] (around 50 %). Despite that, microbead-bound cells (referred as $CD133^+$ cells to simplify) allowed us to mimic the characteristics of GSCs. Since cells were cultured for two weeks before the flow cytometry analysis, the low-expression levels of CD133 can be explained by the probable differentiation of GSC despite the use of a specific stem cell medium designed to repress this process. Contrary to our expectations, $CD133^-$ cells showed a small degree of labeling for both CD133 (10%) and nestin (40 %) (Figures 12 and 13). Traditionally, nestin has been reported for its importance as a neural stem cell marker. However, in the past years, expression of nestin was shown not to be stem cell exclusive, but has also been associated with general proliferation of progenitor cell populations within neoplasms[64,113]. Interestingly, the work of Li Shen and coleagues[113] and Jirina Relichova and colleagues[114] stated that nestin has and heterogeneous expression pattern in glioblastoma cell lines, as observed in our study. Our results can be further justified taking into consideration that not all $nestin^+$ cells are also $CD133^+$ and, therefore, $nestin^+/CD133^-$ cells would not be retain in the magnetic field and would be present in the unbound cell population.

Regarding CD133, this marker has been suggested to be a cancer stem cell marker since only $CD133^+$ cells from brain tumor biopsies were able to initiate brain cancer in mouse models. However, in 2008, Jian Wang and his group demonstrated that $CD133^-$ cells were tumorgenic[115]. With further experiments, these researchers found that tumors derived from CD133 negative cells contained 1–5% CD133 positive cells[115]. These results suggest that even using different isolation methods, there is always the possibility that some $CD133^+$ cells escape the separation protocols.

As anticipated, miRNA profiles of $CD133^+$ and $CD133^-$ cells showed significant and interesting differences (figure 15). MicroRNA-128, in particular, was found to be downregulated in $CD133^+$ cells when compared to $CD133^-$ cells. This microRNA had

previously been reported to be downregulated in GBM. However, our results show that its expression is even more downregulated in $CD133^+$, suggesting that the absence of this miRNA may be important to maintain cancer stem cell properties. E2F3a, a transcription factor that induces the expression of genes involved in cell cycle progression, and Bmi-1, a member of the polycomb repressor complex (PRC1) are two of the main targets of miR-128[35,88,91].

Our results fully agree with the data obtained by Pierpaolo Peruzz and colleagues[105] in 2013, where they showed that miR-128 is an important suppressor of PRC activity in glioma stem cells, and its absence occurs early during gliomagenesis. They showed that besides Bmi-1, a component of PRC1, miR-128 also targets the mRNA of SUZ12, a key component of PRC2. Also in line with our results is the work performed in 2008 by Jakub Godlewski and colleagues. They focused their research on the effects of miR-128 on glioma self-renewal, which is thought to be a characteristic of GBM stem-like cells regulated by Bmi-1. The authors demonstrated that miR-128 specifically blocked glioma self-renewal, in a way consistent with Bmi-1 down-regulation. Altogether, these results suggest that miR-128 absence is essential for GBM self-renewal and resistance to therapy. Taking this into account, upregulating miR-128 could be a promising therapeutic strategy for GBM.

To shed some light on the role of miR-128 in GSCs and GBM biology, we tried to deliver miR-128 mimics to U87 cells and to U87/$CD133^+$ cells. We were able to increase miR-128 expression (figure 16) and decrease the mRNA levels for BMI-1 (figure 17) in U87 cells, but unfortunately, we were unable to do the same in the neurospheres present in U87/ $CD133^+$ cultures.

Figure 18 shows that miR-128 overexpression combined with sunitinib (15µM) was able to reduce U87 cell viability to approximately 20%. This result is similar to that obtained with the double concentration of sunitinib (30µM), and is in agreement with the results obtained by Pedro M. Costa et al[116]. These data suggest that miR-128 overexpression sensitized U87 cells to sunitinib-induced cell death and prove that it is possible achieve a significant reduction in cellular viability employing a lower concentration of the drug, which would probably result in a reduction in the expected side effects.

As stated previously, miRNAs are differentially expressed in normal tissues and cancers, and aberrant miRNA expression is associated with GBM tumorigenesis. For this reason,

these small RNA molecules are very attractive therapeutic targets for GBM. MicroRNA-128 has been the subject of several studies since it is downregulated in several tumor types, such as the breast cancer and GBM. In 2011, a group of researchers led by Yinghua Zhu showed similar results to those obtained in the present study, but in breast cancer. By transfecting breast tumor–initiating cells (BT-IC) with miR-128, they sensitized BT-ICs to the DNA-damaging effects of doxorubicin, illustrating the therapeutic potential of this miRNA. Those findings indicated that Bmi-1 (validated target of miR-128) overexpression is a stem cell–like feature underlying chemotherapy resistance in these cells[117].

Other reports found in the literature focus in several other miRNAs found to be differently expressed in $CD133^+$ cells in this study. In the work develop by Zhen Fu et al and coworkers[13], miR-181b was shown to function as a tumor suppressor, repressing proliferation and reducing chemoresistance to temozolomide in GSCs. The results presented by the authors suggested that the miR-181b could potentially serve as a therapeutic agent for eradicating glioma stem cells[118].

In the same line of research, focusing on miRNA-mediated sensitization of tumor cells, our group has also shown interesting results concerning miR-21. Contrary to what was done in the studies mentioned above, we have used anti-miR-21 oligonucleotides to sensitize U87 cells to sunitinib through miR-21 silencing[116]. All this studies reflect the fact that miRNA-based modulation strategies can also be used to sensitized tumor cells to other treatments and to potentiate the effect of conventional therapies.

In what concerns our inability to modulate miR-128 and BMI-1 expression in U87/ $CD133^+$, these results can be explained by the inherent characteristics of neurosphere cultures. Neurospheres are characterized by a condensed structure of its cells, which can hinder the diffusion of molecules to the innermost cells[119]. This characteristic of neurosphere cultures brings yet another important issue. When neurospheres grow larger the percentage of stem-like cells decreases due to poor diffusion of growth factors and an increase in central hypoxia[119]. Since neurosphere culture presents all this associated limitations, other means for the study and transfection of cancer stem cells are urgently required. In our work we tested two preliminary approaches aiming at improving the transfection of glioma stem cells, based on the use of 1) laminin-coated plates to allow monolayer GSC culture and 2) CTX-SNALP to improve GSC transfection.

Realizing the need for new cancer stem cell culture options, Steven M. Pollard and colleagues[120] first cultured these cells in laminin-coated plates, in order to promote adherence without losing stemness. The adherent GSCs were more homogeneous than neurosphere cultures, and presented high expression of GSC genes, such as Sox2, Nestin, CD133 and CD44. In our study we showed that laminin cultured cells maintain CD133 labeling (figure 19). Culture on an adherent laminin surface allows for a more uniform exposure to growth factors and oxygen. Decreased cell to cell contact and integrin/laminin signaling may also maintain the stem-cell-like state by limiting differentiation signaling[120]. Taking into account that glioma stem cells in laminin-coated wells stay adherent and that lipoplexes and other non-viral delivery systems have the tendency to become deposit due to gravity at the surface of exposed cells, this culture method could help improve transfection of GSCs *in vitro* and to study the therapeutic efficacy of miRNA modulation in these cells.

Stable nucleic acid lipid particles (SNALPs) were shown[111] to be very efficient to deliver small interfering RNAs (siRNAs) to different types of cancer cells. In SNALPs, the siRNA is surrounded by a lipid bilayer containing a mixture of cationic and fusogenic lipids. These complex liposomes are quite versatile and can be coupled with peptides to mediate specific delivery to tumor cells, taking advantage of overexpressed tumor receptors. In this regard, our group has developed CTX-coupled SNALPs to promote both siRNA or anti-miRNA oligonucleotide delivery to glioblastoma cells[111]. Chlorotoxin was reported to bind to matrix metalloproteinase-2, which is upregulated in gliomas and poorly expressed in normal tissues. Taking this into account, this scorpion-derived peptide can be used to enhance SNALP targeting to GBM cells. In the study by Pedro M Costa and colleagues[111], the authors showed that CTX-coupled SNALPs enhance the delivery of anti-miR-21 oligonucleotides to different glioma cell lines and intracranial tumors, with reduced affinity for non-cancer cells[111]. In our study, we were able to increase SNALP internalization in U87/CD133^{+} cells by 55% using CTX as a ligand (figure 20), suggesting that this could be an interesting strategy to mediate the microRNA modulation in GSCs cells.

Overall, our results reflect the current belief that miRNAs play an important role in GBM and that miRNA-modulation strategies, alone or in combination with conventional therapies, may allow a significant improvement in patient care in the a near future.

Chapter 6 Conclusions

6) Conclusions

The results obtained in this work and their implications in the field of gene therapy for glioblastoma multiforme (GBM) and glioma stem cells (GSCs) led to several interesting conclusions that are summarized below.

- Glioma stem cells isolated from the U87 cell line (U87/CD133^{+} cells) and maintained in culture in non-adherent conditions, express both nestin and CD133 two weeks after isolation. U87/CD133^{+} cells, contrarily to U87/CD133^{-} cells, are able to form neurospheres in these conditions.

- When compared directly, U87/CD133^{+} and U87/CD133^{-} cells show different miRNA expression profiles. MiR-128 was shown to be downregulated in GSCs, and, importantly, overexpression of miR-128 was able to sensitize U87 cells to sunitinib-induced cell death.

- Laminin-coated plates, due to its adherent capacity, can be an interesting new cancer stem cell culture method for miRNA transfection. Moreover, CTX-SNALPs showed increased internalization compared to NT-SNALPs and can be another strategy to improve the delivery of small interfering RNAs and miRNA mimics to GSCs.

Chapter 7 References

1. Louis, D. N. *et al.* The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol.* **114,** 97–109 (2007).

2. Adamson, C. *et al.* Glioblastoma multiforme: a review of where we have been and where we are going. *Expert Opin. Investig. Drugs* **18,** 1061–83 (2009).

3. Schwartzbaum, J. *et al*. Epidemiology and molecular pathology of glioma. *Nat. Clin. Pract. Neurol.* **2,** 494–503; quiz 1 p following 516 (2006).

4. Furnari, F. B. *et al.* Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes Dev.* **21,** 2683–710 (2007).

5. Ohgaki, H. & Kleihues, P. The definition of primary and secondary glioblastoma. *Clin. Cancer Res.* **19,** 764–72 (2013).

6. Ohgaki, H. & Kleihues, P. Genetic pathways to primary and secondary glioblastoma. *Am. J. Pathol.* **170,** 1445–53 (2007).

7. Nakada, M. *et al.* Aberrant Signaling Pathways in Glioma. 3242–3278 (2011). doi:10.3390/cancers3033242

8. Ware, M. L., Berger, M. S. & Binder, D. K. Molecular biology of glioma tumorigenesis. *Histol. Histopathol.* **18,** 207–16 (2003).

9. Vauleon, E., Avril, T., Collet, B., Mosser, J. & Quillien, V. Overview of cellular immunotherapy for patients with glioblastoma. *Clin. Dev. Immunol.* **2010,** (2010).

10. Huang, Z., Cheng, L., Guryanova, O. a, Wu, Q. & Bao, S. Cancer stem cells in glioblastoma--molecular signaling and therapeutic targeting. *Protein Cell* **1,** 638–55 (2010).

11. Soltysova, A., Altanerova, V. & Altaner, C. Cancer stem cells *. 435–440 (2005).

12. Bao, S. *et al.* Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444,** 756–60 (2006).

13. Alison, M. R., Lin, W.-R., Lim, S. M. L. & Nicholson, L. J. Cancer stem cells: in the line of fire. *Cancer Treat. Rev.* **38,** 589–98 (2012).

14. Rev, A., Mech, P., Downloaded, D. & Louis, D. N. Molecular Pathology of Malignant Gliomas. (2006). doi:10.1146/annurev.pathol.1.110304.100043

15. Nakada, M. *et al.* Aberrant Signaling Pathways in Glioma. 3242–3278 (2011). doi:10.3390/cancers3033242

16. Puputti, M. *et al.* Amplification of KIT, PDGFRA, VEGFR2, and EGFR in gliomas. *Mol. Cancer Res.* **4,** 927–34 (2006).

17. Hynes, R. O. Integrins : Bidirectional , Allosteric Signaling Machines In their roles as major adhesion receptors , integrins. **110,** 673–687 (2002).

18. Kawataki, T. *et al.* Laminin isoforms and their integrin receptors in glioma cell migration and invasiveness: Evidence for a role of alpha5-laminin(s) and alpha3beta1 integrin. *Exp. Cell Res.* **313,** 3819–31 (2007).

19. Xu, Y., Stamenkovic, I. & Yu, Q. CD44 attenuates activation of the hippo signaling pathway and is a prime therapeutic target for glioblastoma. *Cancer Res.* **70,** 2455–64 (2010).

20. Bayin, N. S., Modrek, A. S. & Placantonakis, D. G. Glioblastoma stem cells: Molecular characteristics and therapeutic implications. *World J. Stem Cells* **6,** 230–238 (2014).

21. Zhang, X., Zhang, W., Cao, W.-D., Cheng, G. & Zhang, Y.-Q. Glioblastoma multiforme: Molecular characterization and current treatment strategy (Review). *Exp. Ther. Med.* **3,** 9–14 (2012).

22. Eyler, C. E. & Rich, J. N. Looking in the miR-ror : TGF-β – mediated activation of NF-κB in glioma. 3473–3475 (2012). doi:10.1172/JCI66058.play

23. Lebelt, A. *et al.* Angiogenesis in gliomas. *Folia Histochem. Cytobiol.* **46,** 69–72 (2008).

24. Chamberlain, M. C., Glantz, M. J., Chalmers, L., Van Horn, A. & Sloan, A. E. Early necrosis following concurrent Temodar and radiotherapy in patients with glioblastoma. *J. Neurooncol.* **82,** 81–3 (2007).

25. Deeken, J. F. & Löscher, W. The blood-brain barrier and cancer: transporters, treatment, and Trojan horses. *Clin. Cancer Res.* **13,** 1663–74 (2007).

26. Dresemann, G. Temozolomide in malignant glioma. *Onco. Targets. Ther.* **3,** 139–46 (2010).

27. Hirst, T. C. *et al.* Systematic review and meta-analysis of temozolomide in animal models of glioma: was clinical efficacy predicted? *Br. J. Cancer* **108,** 64–71 (2013).

28. Czabanka, M., Vinci, M., Heppner, F., Ullrich, A. & Vajkoczy, P. Effects of sunitinib on tumor hemodynamics and delivery of chemotherapy. *Int. J. Cancer* **124,** 1293–300 (2009).

29. Zhou, Q. & Gallo, J. M. Differential effect of sunitinib on the distribution of temozolomide in an orthotopic glioma model. *Neuro. Oncol.* **11,** 301–10 (2009).

30. De Boüard, S. *et al.* Antiangiogenic and anti-invasive effects of sunitinib on experimental human glioblastoma. *Neuro. Oncol.* **9,** 412–23 (2007).

31. Weller, M. Immunotherapy for glioblastoma: a long and winding road. *Neuro. Oncol.* **12,** 319 (2010).

32. Mohme, M., Neidert, M. C., Regli, L., Weller, M. & Martin, R. Immunological challenges for peptide-based immunotherapy in glioblastoma. *Cancer Treat. Rev.* **40,** 248–58 (2014).

33. Xu, L. W., Chow, K. K. H., Lim, M. & Li, G. Current vaccine trials in glioblastoma: a review. *J. Immunol. Res.* **2014,** 796856 (2014).

34. Savio, L. C. Gene Therapy for Brain Tumors: Regression of experimental gliomas by adenovirus-mediated gene transfer in vivo. **91,** 3054–3057 (1994).

35. Skalsky, R. L. & Cullen, B. R. Reduced expression of brain-enriched microRNAs in glioblastomas permits targeted regulation of a cell death gene. *PLoS One* **6,** e24248 (2011).

36. Tu, Y. *et al.* MicroRNA-218 inhibits glioma invasion, migration, proliferation, and cancer stem-like cell self-renewal by targeting the polycomb group gene Bmi1. *Cancer Res.* **73,** 6046–55 (2013).

37. Kefas, B. *et al.* microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma. *Cancer Res.* **68,** 3566–72 (2008).

38. Clevers, H. The cancer stem cell: premises, promises and challenges. *Nat. Med.* **17,** 313–9 (2011).

39. Dalerba, P., Cho, R. W. & Clarke, M. F. Cancer stem cells: models and concepts. *Annu. Rev. Med.* **58,** 267–84 (2007).

40. Visvader, J. E. & Lindeman, G. J. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat. Rev. Cancer* **8,** 755–68 (2008).

41. Guo, W., Lasky, J. L. & Wu, H. Cancer stem cells. *Pediatr. Res.* **59,** 59R–64R (2006).

42. Natsume, A. *et al.* Glioma-initiating cells and molecular pathology: implications for therapy. *Brain Tumor Pathol.* **28,** 1–12 (2011).

43. Lobo, N. a, Shimono, Y., Qian, D. & Clarke, M. F. The biology of cancer stem cells. *Annu. Rev. Cell Dev. Biol.* **23,** 675–99 (2007).

44. Liu, S. *et al.* Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res.* **66,** 6063–71 (2006).

45. Sayed, S. I. *et al.* Implications of understanding cancer stem cell (CSC) biology in head and neck squamous cell cancer. *Oral Oncol.* **47,** 237–43 (2011).

46. Valkenburg, K. C., Graveel, C. R., Zylstra-Diegel, C. R., Zhong, Z. & Williams, B. O. Wnt/β-catenin Signaling in Normal and Cancer Stem Cells. *Cancers (Basel).* **3,** 2050–79 (2011).

47. Wang, R. *et al.* Glioblastoma stem-like cells give rise to tumour endothelium. *Nature* **468,** 829–33 (2010).

48. Li, C. *et al.* Identification of pancreatic cancer stem cells. *Cancer Res.* **67,** 1030–7 (2007).

49. Bayin, N. S., Modrek, A. S. & Placantonakis, D. G. Glioblastoma stem cells: Molecular characteristics and therapeutic implications. *World J. Stem Cells* **6,** 230–238 (2014).

50. Rich, J. N. Cancer stem cells in radiation resistance. *Cancer Res.* **67,** 8980–4 (2007).

51. Clarke, M. F. *et al.* Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res.* **66,** 9339–44 (2006).

52. Joo, K. M. *et al.* MET signaling regulates glioblastoma stem cells. *Cancer Res.* **72,** 3828–38 (2012).

53. Yu, S. *et al.* Isolation and characterization of cancer stem cells from a human glioblastoma cell line U87. **265,** 124–134 (2008).

54. Liu, G. *et al.* Analysis of gene expression and chemoresistance of CD133 + cancer stem cells in glioblastoma. **12,** 1–12 (2006).

55. Magee, J. a, Piskounova, E. & Morrison, S. J. Cancer stem cells: impact, heterogeneity, and uncertainty. *Cancer Cell* **21,** 283–96 (2012).

56. Lugli, a *et al.* Prognostic impact of the expression of putative cancer stem cell markers CD133, CD166, CD44s, EpCAM, and ALDH1 in colorectal cancer. *Br. J. Cancer* **103,** 382–90 (2010).

57. Medema, J. P. Cancer stem cells: the challenges ahead. *Nat. Cell Biol.* **15,** 338–44 (2013).

58. Croker, A. K. *et al.* High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability. *J. Cell. Mol. Med.* **13,** 2236–52 (2009).

59. Eramo, a *et al.* Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ.* **15,** 504–14 (2008).

60. Bonnet D, *et al.* Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Publishing Group.* **3,** 730-37 (1997)

61. Brescia, P. *et al.* CD133 is essential for glioblastoma stem cell maintenance. *Stem Cells* **31,** 857–69 (2013).

62. Beier, D. *et al.* CD133 + and CD133 À Glioblastoma-Derived Cancer Stem Cells Show Differential Growth Characteristics and Molecular Profiles. 4010–4015 (2007).

63. Park, D. *et al.* Nestin is required for the proper self-renewal of neural stem cells. *Stem Cells* **28,** 2162–71 (2010).

64. Wiese, C. *et al.* Nestin expression--a property of multi-lineage progenitor cells? *Cell. Mol. Life Sci.* **61,** 2510–22 (2004).

65. Heddleston, J. M. *et al.* Hypoxia inducible factors in cancer stem cells. *Br. J. Cancer* **102,** 789–95 (2010).

66. Bao, S. *et al.* Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. *Cancer Res.* **66,** 7843–8 (2006).

67. Castel, S. E. & Martienssen, R. a. RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. *Nat. Rev. Genet.* **14,** 100–12 (2013).

68. Esteller, M. Non-coding RNAs in human disease. *Nat. Rev. Genet.* **12,** 861–74 (2011).

69. Chitwood, D. H. & Timmermans, M. C. P. Small RNAs are on the move. *Nature* **467,** 415–9 (2010).

70. Wilson, R. C. & Doudna, J. a. Molecular mechanisms of RNA interference. *Annu. Rev. Biophys.* **42,** 217–39 (2013).

71. Berezikov, E. Evolution of microRNA diversity and regulation in animals. *Nat. Rev. Genet.* **12,** 846–60 (2011).

72. Filipowicz, W., Bhattacharyya, S. N. & Sonenberg, N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat. Rev. Genet.* **9,** 102–14 (2008).

73. Winter, J., Jung, S., Keller, S., Gregory, R. I. & Diederichs, S. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat. Cell Biol.* **11,** 228–34 (2009).

74. Gu, S. & Kay, M. a. How do miRNAs mediate translational repression? *Silence* **1,** 11 (2010).

75. Alerting, E. MicroRNA function : Multiple mechanisms for a tiny RNA ? MicroRNA function : Multiple mechanisms for a tiny RNA ? 1753–1761 (2005).

76. Sibley, C. R. *et al.* The biogenesis and characterization of mammalian microRNAs of mirtron origin. *Nucleic Acids Res.* **40,** 438–48 (2012).

77. Carthew, R. W. & Sontheimer, E. J. Origins and Mechanisms of miRNAs and siRNAs. *Cell* **136,** 642–55 (2009).

78. Cai, Y., Yu, X., Hu, S. & Yu, J. A brief review on the mechanisms of miRNA regulation. *Genomics. Proteomics Bioinformatics* **7,** 147–54 (2009).

79. Feng, W. & Feng, Y. MicroRNAs in neural cell development and brain diseases. *Sci. China. Life Sci.* **54,** 1103–12 (2011).

80. Sana, J., Hajduch, M., Michalek, J., Vyzula, R. & Slaby, O. MicroRNAs and glioblastoma: roles in core signalling pathways and potential clinical implications. *J. Cell. Mol. Med.* **15,** 1636–44 (2011).

81. Li, Y. *et al.* Comprehensive analysis of the functional microRNA-mRNA regulatory network identifies miRNA signatures associated with glioma malignant progression. *Nucleic Acids Res.* **41,** e203 (2013).

82. Aldaz, B. *et al.* Involvement of miRNAs in the Differentiation of Human Glioblastoma Multiforme Stem-Like Cells. **8,** 1–15 (2013).

83. Calin, G. A. & Croce, C. M. MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res.* **66,** 7390–4 (2006).

84. Lawler, S. & Chiocca, E. A. Emerging functions of microRNAs in glioblastoma. *J. Neurooncol.* **92,** 297–306 (2009).

85. Liu, C. & Tang, D. G. MicroRNA regulation of cancer stem cells. *Cancer Res.* **71,** 5950–4 (2011).

86. Ortensi, B., Setti, M., Osti, D. & Pelicci, G. Cancer stem cell contribution to glioblastoma invasiveness. *Stem Cell Res. Ther.* **4,** 18 (2013).

87. Li, M. *et al.* MicroRNA in Human Glioma. *Cancers (Basel).* **5,** 1306–31 (2013).

88. Møller, H. G. *et al.* A systematic review of microRNA in glioblastoma multiforme: micro-modulators in the mesenchymal mode of migration and invasion. *Mol. Neurobiol.* **47,** 131–44 (2013).

89. Zhang, Y. *et al.* MicroRNA-128 inhibits glioma cells proliferation by targeting transcription factor E2F3a. *J. Mol. Med. (Berl).* **87,** 43–51 (2009).

90. Peruzzi, P. *et al.* MicroRNA-128 coordinately targets Polycomb Repressor Complexes in glioma stem cells. *Neuro. Oncol.* **15,** 1212–24 (2013).

91. Karsy, M., Arslan, E. & Moy, F. Current Progress on Understanding MicroRNAs in Glioblastoma Multiforme. *Genes Cancer* **3,** 3–15 (2012).

92. Sasayama, T., Nishihara, M., Kondoh, T., Hosoda, K. & Kohmura, E. MicroRNA-10b is overexpressed in malignant glioma and associated with tumor invasive factors, uPAR and RhoC. *Int. J. Cancer* **125,** 1407–13 (2009).

93. Guessous, F. *et al.* Oncogenic effects of miR-10b in glioblastoma stem cells. *J. Neurooncol.* **112,** 153–63 (2013).

94. Zhang, C.-Z. *et al.* MiR-221 and miR-222 target PUMA to induce cell survival in glioblastoma. *Mol. Cancer* **9,** 229 (2010).

95. Krichevsky, A. M. & Gabriely, G. miR-21: a small multi-faceted RNA. *J. Cell. Mol. Med.* **13,** 39–53 (2009).

96. Papagiannakopoulos, T., Shapiro, A. & Kosik, K. S. MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. *Cancer Res.* **68,** 8164–72 (2008).

97. Gabriely, G. *et al.* MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. *Mol. Cell. Biol.* **28,** 5369–80 (2008).

98. Huse, J. T. *et al.* The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis in vivo. *Genes Dev.* **23,** 1327–37 (2009).

99. Theiss, A. L. & Sitaraman, S. V. The role and therapeutic potential of prohibitin in disease. *Biochim. Biophys. Acta* **1813,** 1137–43 (2011).

100. Qian, X. *et al.* MicroRNA-26a promotes tumor growth and angiogenesis in glioma by directly targeting prohibitin. *CNS Neurosci. Ther.* **19,** 804–12 (2013).

101. Godlewski, J. *et al.* MicroRNA-451 regulates LKB1/AMPK signaling and allows adaptation to metabolic stress in glioma cells. *Mol. Cell* **37,** 620–32 (2010).

102. Gabriely, G. *et al.* Human glioma growth is controlled by microRNA-10b. *Cancer Res.* **71,** 3563–72 (2011).

103. Aldaz, B. *et al.* Involvement of miRNAs in the Differentiation of Human Glioblastoma Multiforme Stem-Like Cells. *PLoS One* **8,** e77098 (2013).

104. Gartel, A. L. & Kandel, E. S. miRNAs: Little known mediators of oncogenesis. *Semin. Cancer Biol.* **18,** 103–10 (2008).

105. Peruzzi, P. *et al.* MicroRNA-128 coordinately targets Polycomb Repressor Complexes in glioma stem cells. *Neuro. Oncol.* **15,** 1212–24 (2013).

106. Ciafrè, S. a *et al.* Extensive modulation of a set of microRNAs in primary glioblastoma. *Biochem. Biophys. Res. Commun.* **334,** 1351–8 (2005).

107. Silber, J. *et al.* miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. *BMC Med.* **6,** 14 (2008).

108. Lebrun, D. G. & Li, M. MicroRNAs in Glioblastoma Multiforme : Profiling Studies and Therapeutic Impacts. **3,** 93–105 (2011).

109. Guidi, M. *et al.* Overexpression of miR-128 specifically inhibits the truncated isoform of NTRK3 and upregulates BCL2 in SH-SY5Y neuroblastoma cells. *BMC Mol. Biol.* **11,** 95 (2010).

110. Zhu, Y. *et al.* Reduced miR-128 in breast tumor-initiating cells induces chemotherapeutic resistance via Bmi-1 and ABCC5. *Clin. Cancer Res.* **17,** 7105–15 (2011).

111. Costa, P. M. *et al.* Tumor-targeted Chlorotoxin-coupled Nanoparticles for Nucleic Acid Delivery to Glioblastoma Cells: A Promising System for Glioblastoma Treatment. *Mol. Ther. Nucleic Acids* **2,** e100 (2013).

112. Singh, S. K. *et al.* Identification of a Cancer Stem Cell in Human Brain Tumors Identification of a Cancer Stem Cell in Human Brain Tumors. 5821–5828 (2003).

113. Lu, W. J. *et al.* Inducible expression of stem cell associated intermediate filament nestin reveals an important role in glioblastoma carcinogenesis. *Int. J. Cancer* **128,** 343–51 (2011).

114. Veselska, R. *et al.* Nestin expression in the cell lines derived from glioblastoma multiforme. *BMC Cancer* **6,** 32 (2006).

115. Wang, J. *et al.* CD133 negative glioma cells form tumors in nude rats and give rise to CD133 positive cells. *Int. J. Cancer* **122,** 761–8 (2008).

116. Costa, P. M. *et al.* MicroRNA-21 silencing enhances the cytotoxic effect of the antiangiogenic drug sunitinib in glioblastoma. *Hum. Mol. Genet.* **22,** 904–18 (2013).

117. Zhu, Y., Yu, F. & Jiao, Y. Reduced miR-128 in Breast Tumor − Initiating Cells Induces Chemotherapeutic Resistance via Bmi-1 and ABCC5. 7105–7115 (2011). doi:10.1158/1078-0432.CCR-11-0071

118. Li, P. *et al.* MiR-181b suppresses proliferation of and reduces chemoresistance to temozolomide in U87 glioma stem cells. *J. Biomed. Res.* **24,** 436–43 (2010).

119. Woolard, K. & Fine, H. a. Glioma stem cells: better flat than round. *Cell Stem Cell* **4,** 466–7 (2009).

120. Pollard, S. M. *et al.* Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. *Cell Stem Cell* **4,** 568–80 (2009).

121. Tanaka, S., Louis, D. N., Curry, W. T., Batchelor, T. T. & Dietrich, J. Diagnostic and therapeutic avenues for glioblastoma: no longer a dead end? *Nat. Rev. Clin. Oncol.* **10,** 14–26 (2013).

122. Jordan, C. T., Guzman, M. L. & Noble, M. Cancer stem cells. *N. Engl. J. Med.* **355,** 1253–61 (2006).