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

“Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning”

Albert Einstein

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Contents

Abbreviations

3'UTR	3' Untranslated region
AML	Acute myeloid leukemia
ALDH	Aldehyde dehydrogenase
AIC	5-aminoimidazole-4-carboxamide
AGO	Argonaute protein
AKT	Protein kinase B
B-CLL	B- cell chronic lymphocytic leukemia
Bcl-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
BBB	Blood-brain barrier
Bmi1	B-lymphoma Mo-MLV insertion region 1 homolog
BMP	Bone morphogenetic proteins
BCRP1	Breakpoint cluster region pseudogene 1
CSC	Cancer stem cell
CDC42	Cell division control protein 42 homolog
cIAP	Cellular inhibitor of apoptosis protein-1
CNS	Central nervous system
CTX	Chlorotoxin
CDK6	Cyclin-dependent kinase
CTGF	Connective tissue growth factor
CT	X-ray computed tomography
CXCR4	C-X-C chemokine receptor type 4
DNMT1	DNA methyltransferase 1
DNMT3A	DNA methyltransferase 3A
DNMT3B	DNA methyltransferase 3B
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

EMT	Epithelial-mesenchymal transition
FAK	Focal adhesion kinase pathway
FGF	Basic fibroblast growth factor
GSC	Glioblastoma stem-like cell
HAT	Histone acetyltransferase
LOH	Loss of heterozygosity
MRI	Magnetic resonance imaging
MMP	Matrix-degrading metalloproteinase
MAGEA1	Melanoma associated antigen 1
MGMT	Methylguanine-DNA methyltransferase
MiRNA	MicroRNA
MMR	DNA mismatch repair system
MAPK/ERK	Mitogen-activated protein kinase pathway
MTIC	Monomethyl triazene 5-(3-methyltriazen-1-yl)-imidazole-4-carboxamide
NSC	Neural stem cells
O^6-MeG	O^6- methylguanine
Oct-4	Octamer-binding transcription factor 4
OLIG2	Oligodendrocyte transcription factor 2
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PTEN	Phosphatase and tensin homolog
PI3k/AKT	Phosphatidylinositol 3'-kinase
PGRN	Progranulin
RB	Retinoblastoma
RISC	RNA-induced silencing complex
RTK	Tyrosine kinase receptors
Sox2	Sex determining region Y-box 2
SiRNA	Small interference RNA
SNALP	Stable nucleic acid lipid particle

TMZ	Temozolomide
THBS1	Thrombospondin 1
TP53	Tumor protein 53
VEGF	Vascular endothelial growth factor
WHO	World health organization
XAF-1	Xiap-associated factor 1
YY1	Ying Yang 1 transcription factor
ZEB	zinc-finger E-box-binding homeobox transcription factor

Abstract

Abstract

Glioblastoma (GBM) is the most common and aggressive primary brain tumor. Despite recent advances in the identification of genetic and molecular alterations that drive GBM pathogenesis, patient outcome has not significantly improved over the last decade. Standard treatment consists in a combined therapy, which includes surgical resection followed by radiotherapy and concomitant or adjuvant chemotherapy with temozolomide. However, despite being aggressive, this approach lacks efficiency mainly due to the rapid and infiltrative growth of tumor cells, their resistance to apoptosis and extensive cellular heterogeneity, which result in a median survival time of only 12 to 15 months from the time of diagnosis. Therefore, there is an urgent need to develop new therapeutic options that aim not only to delay tumor progression and improve the overall survival of each patient, but also reduce the side effects common to most anti-cancer treatments.

The recent identification of a $CD133^+$ subpopulation of cells, within the tumor mass, that exhibit stem-like properties, such as unlimited replicative potential and the ability to differentiate and give rise to the bulk of tumor, both *in vitro* and *in vivo*, opened new doors to a better understanding of GBM pathogenesis and established a new promising target for more effective therapies. Targeting these cells is now believed to be the key approach to a complete tumor eradication, since they are largely responsible for the acquired resistance to standard radio- and chemotherapy that often results in tumor recurrence.

In addition, deregulated microRNAs are also emerging as important regulators of gliomagenesis, playing key roles in many biological processes that drive tumor initiation and progression, including cell proliferation, invasion and migration, resistance to apoptosis, and maintenance of glioblastoma stem-like cells properties (GSCs), thus suggesting that gene therapy approaches based on miRNA modulation might represent an effective therapeutic strategy for GBM treatment.

In this work, our major goal was to isolate and characterize $CD133^+$ GSCs isolated from both a primary and a recurrent human GBM cell lines, U87MG and DBTRG-05MG, respectively, and further evaluate their expression profile concerning miR-29b, a tumor suppressive miRNA closely related to aberrant epigenetic mechanisms, as well as GBM enhanced cell migration and invasion.

We also proposed to modulate the expression of miR-29b and investigate whether this strategy could sensitize GBM cells to chemotherapy and impact global DNA methylation status.

The obtained results demonstrated that DBTRG $CD133^+$ cells, isolated by magnetic cell sorting, exhibit a more pronounced stem phenotype, and express less miR-29b in comparison with the $CD133^+$ fraction isolated from U87 cells. Also, miR-29b expression could be inversely correlated with the expression of progranulin, DNA methyltransferases 3A and 3B, as well as the transcription factor Ying Yang 1, which constitute validated targets of this microRNA. In addition, we further confirmed that miR-29b expression is downregulated in both CD133-positive and negative fractions isolated from the two GBM cell lines, as compared to human astrocytes.

Reestablishment of miR-29b expression levels, using a commercial reagent as a delivery vector, enhanced cell sensitivity of both U87 and DBTRG cells to the cytotoxic effects of sunitinib and axitinib, two tyrosine kinase inhibitors currently under phase II clinical trials for GBM treatment, but failed to show any effect when combined with the standard treatment drug temozolomide. Furthermore, miR-29b modulation also resulted in a decrease in the global DNA methylation status of DBTRG cells, evaluated through quantification of 5-mC levels, while showing no visible effect in U87 global DNA methylation signature.

Overall our results suggest that miR-29b might play a crucial role in GBM progression, and therefore, development of a targeted therapy based on reestablishment of its expression levels might prove to be an effective approach towards GBM treatment.

Keywords: Glioblastoma; Glioblastoma stem-like cells; miR-29b; Chemoresistance; Epigenetic signature.

Resumo

Resumo

O glioblastoma (GBM) é o tumor cerebral primário mais comum e mais agressivo nos adultos. Apesar de se terem feito recentemente grandes avanços no sentido de identificar mecanismos genéticos e moleculares desregulados em GBM e responsáveis por impulsionar o desenvolvimento e progressão deste tipo de tumor, a esperança média de vida dos pacientes afetados continua muito baixa, não se tendo registado melhorias significativas nos últimos anos.

O tratamento convencional, na sua maioria paliativo, consiste numa terapia combinada que incluiu a remoção cirúrgica da massa tumoral principal, seguida de radioterapia e quimioterapia com o agente quimioterapêutico temozolomide (TMZ). No entanto, apesar de ser bastante agressiva, esta abordagem terapêutica é pouco eficiente e o tumor reaparece na maioria dos casos. De facto, a grande capacidade proliferativa e invasiva das células tumorais de GBM, aliadas à heterogeneidade celular e acima de tudo à elevada capacidade de resistência à apoptose, resultam na falha do tratamento e apenas conferem um tempo médio de vida que varia entre os 12 e 15 meses após o diagnóstico.

Assim, é urgente o desenvolvimento de novas terapias que visem não só o atraso na progressão tumoral e o aumento da sobrevivência média de pacientes com GBM, mas também a redução dos efeitos secundários tão comuns neste tipo de tratamentos anticancerígenos.

A recente identificação de uma subpopulação de células tumorais que expressam o marcador CD133 e que apresentam propriedades comuns a células estaminais, abriu novas portas para melhor compreender a patogénese deste tumor cerebral e, acima de tudo, estabeleceu um novo alvo terapêutico para terapias mais eficientes e seletivas. Estas células apresentam uma capacidade replicativa ilimitada, assim como a capacidade de se diferenciarem em todos os tipos de células que constituem a massa tumoral principal. Para além disso, esta população é altamente resistente às terapias convencionais. Desta forma, o desenvolvimento de uma terapia direcionada para estas células estaminais de glioblastoma (GSCs) poderá constituir a chave para uma completa erradicação do tumor, evitando assim, a recorrência do mesmo.

Aliada a esta descoberta, os microRNAS cuja expressão se encontra desregulada em GBM, têm emergido com reguladores da iniciação e progressão deste tipo de tumor, desempenhando papeis essenciais numa vasta gama de processos biológicos, que vão desde a proliferação celular, invasão e migração, resistência à apoptose, até à manutenção das propriedades das

células estaminais de GBM. Estas observações sugerem que a modulação da expressão de certos miRNAs poderá também ser uma possível abordagem terapêutica para o tratamento do GBM.

Neste estudo, o nosso principal objetivo foi isolar e caracterizar GSCs, isoladas a partir de uma linha primária e de uma linha recorrente de GBM, U87MG e DBTRG-05MG, respetivamente, com base na expressão de CD133, um marcador de superfície, assim como avaliar a expressão do microRNA- 29b, um microRNA supressor tumoral relacionado com alterações epigenéticas em GBM e com a potenciação de fenómenos de migração e invasão.

Decidimos ainda investigar se uma modulação da expressão deste microRNA era capaz de sensibilizar as células tumorais para a ação da quimioterapia e qual seria os seu impacto na extensão de metilação global do DNA.

Os resultados obtidos demonstraram que as células $CD133^{+}$ isoladas a partir da linha DBTRG exibem um fenótipo de estaminalidade mais pronunciado em comparação com a mesma subpopulação isolada da linha U87. Para além disso, expressam menos miR-29b, o que pode ser inversamente correlacionado com a expressão de progranulina, das DNA metiltransferases 3A e 3B e do fator de transcrição Ying Yang 1, sendo estas proteínas alvos validados deste microRNA. Os nossos resultados mostraram ainda que o miR-29b se encontra subexpresso em ambas as frações $CD133^{+}$ e $CD133^{-}$ isoladas das duas linhas celulares, em comparação com astrócitos humanos.

O restabelecimento dos níveis de miR-29b, usando um reagente comercial como vetor de entrega do microRNA às células tumorais, sensibilizou as duas linhas celulares para os efeitos citotóxicos causados pelo sunitinib e axitinib, dois inibidores de tirosina cinase que estão em fase II de ensaios clínicos para avaliação do seu potencial como agentes quimioterapêutico para o tratamento do GBM. No entanto, a modulação do miR-29b pareceu não surtir efeitos quando combinada com a administração de temozolomide às células.

Por fim, sobreexpressão do miR-29b resultou numa redução dos níveis de metilação global do DNA nas células DBTRG, enquanto que na linha U87 já não se verificou nenhum efeito ao nível do conteúdo de 5-metilcitosina.

No geral, os resultados obtidos neste estudo sugerem que o microRNA 29b desempenha um papel crucial na progressão do GBM e, como tal, o desenvolvimento de uma terapia baseada no restabelecimento dos seus níveis de expressão poderá revelar-se uma abordagem eficiente para o tratamento do GBM.

Palavras-chave: Glioblastoma; Células estaminais de glioblastoma, microRNA-29b; resistência quimioterapêutica; assinatura epigenética.

CHAPTER 1 – Introduction

1.1 Cancer – Overview

Cancer is the leading cause of death in economically developed countries and the second in developing countries.[1] World Health Organization (WHO) estimated approximately 14 million new cases worldwide and 8.2 million cancer related deaths in 2012, with an expected rise by 70% over the next two decades.[2]

A major goal of cancer research is to understand how to counteract the mechanisms that dictate cancer malignancy.[3] Taken that into account, Hanahan and Weinberg (2011) proposed the main hallmarks of cancer, which together dictate malignant growth and contribute to further our understanding of the complex biology of this disease: 1) ability to sustain proliferative signaling, 2) evasion of programmed cell death and growth suppressors, 3) limitless replicative potential, 4) sustained angiogenesis, 5) tissue invasion and metastasis, 6) development of genomic instability, 7) reprogramming energy metabolism and, finally 6) tumor-induced inflammation.[4,5]

Nevertheless, although progress has been made towards reducing cancer incidence and improving overall survival, this disease still accounts for more deaths than any other, emphasizing the urgency to develop new approaches aiming to improve cancer prevention, early detection and treatment.

1.2 Glioblastoma

Gliomas are infiltrative tumors originating from, glial cells that account for 30% of all brain tumors and 80% of primary malignant brain tumors.[6,7]

Based on histological morphology, gliomas are subdivided in astrocytomas, oligodendrogliomas, ependymomas, and mixed gliomas.[7] According to the 2007 WHO classification of tumors of the central nervous system (CNS), gliomas are further categorized into four histologic grades based on their malignancy and aggressiveness, ranging from I to IV.[8,9]

Grade I applies to tumors with low proliferative potential that can be treated with surgical resection. Grade II tumors present infiltrative nature and, despite low-level proliferative activity, often recur and evolve to higher grades of malignancy (low-grade diffuse astrocytoma may recur as anaplastic astrocytoma and glioblastoma). Grade III tumors exhibit histological evidence of malignancy, such as nuclear atypia, brisk mitotic activity and higher vessel density, which often requires treatment with adjuvant radiation and/or chemotherapy.[10]

Grade IV glioma, also known as glioblastoma (GBM), is the most frequent and malignant primary brain tumor, accounting for more than 50% of all gliomas.[11] Histologically, GBM is characterized by rapid, diffuse and infiltrative growth, resistance to apoptosis, and high level of cellular heterogeneity.[12]

Even though GBM is a rare tumor, with a global incidence rate of only 3 to 5 newly diagnosed cases per 100,000 individuals, this tumor has a dramatic outcome for affected patients, providing a median survival time of only 12 to 15 months following diagnosis.[13]

1.2.1 Epidemiology and etiology

GBM may present itself at any age, although it occurs more frequently in adults. The median age at diagnosis is 64 years, with more than 80% of diagnosed patients being older than 55 years and only 1% younger than 20 years. There is a preponderance in males, with an incidence rate 1.5 times higher than in females and twice as high in European descendants as compared to African American or Asian descendants.[13,14]

Furthermore, GBM has a greater incidence among developed countries compared to less developed ones, mainly due to limited access to health care, variations in diagnostic practices, and incomplete cancer reporting.[14]

While the incidence of GBM has slightly increased over the past 20 years, mostly as a result of improved radiologic diagnosis but also due to a true rise in the number of affected patients, the predisposing factors are still poorly understood.[15] So far, the only established environmental risk factors are exposure to high-dose ionizing radiation and inherited mutations of highly penetrant genes associated with rare syndromes (observed in 5-10 % of GBM cases), such as neurofibromatosis 1 and 2, tuberous sclerosis, retinoblastoma 1, Li-Fraumeni syndrome, as well as Turcot's syndrome and multiple hamartoma.[6,11,14–16]

Several epidemiological studies have further reported an association between increased glioma risk and other environmental factors, including severe head injury, dietary risk factors and occupational risk factors, nonetheless none of these are established causes for GBM. On the other hand, asthma, and similar allergic conditions decrease GBM risk, suggesting that immune surveillance may have a role in malignant glioma pathogenesis.[6]

1.2.2 Pathology

1.2.2.1 GBM genesis

GBM may develop *de novo* (primary glioblastoma) or slowly through progression from low-grade diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III).

Although phenotypically indistinguishable, primary and secondary GBM constitute distinct disease subtypes that affect patients of different age, develop through different genetic pathways, and may differ in their response to radio- and chemotherapy.[17] GBM most commonly occurs as a primary tumor, while secondary tumors only account for 5% of all malignant gliomas.[18]

Primary tumors typically affect older patients with a mean age of diagnosis around 62 years and are genetically characterized by loss of heterozygosity (LOH) of chromosome 10q, epidermal growth factor receptor (EGFR) overexpression, deletion of the p16 gene and PTEN mutations. Secondary tumors tend to develop in younger patients, with a mean age of 45 years, and display TP53 mutations (already present in 60% of precursor low-grade astrocytomas), as well as LOH of chromosome 10q, and abnormalities in the pathway regulating the tumor suppressor RB (retinoblastoma).[19]

1.2.2.2 Clinical presentation and diagnosis

GBM cells are usually located in the subcortical white matter of the cerebral hemispheres and frequently extend from the frontal lobe into the temporal lobe. The high infiltrative nature of these cells, allows them to invade the adjacent cortex and progress through the corpus callosum into the contralateral hemisphere.[8]

Tumor growth and invasion of adjacent normal tissues often results in raised intracranial pressure, leading to headaches, nausea and papilledema. Apart from these, patients may also experience focal neurological deficits, confusion, memory loss, personality changes and seizures.[14,15] Nevertheless GBM symptoms usually appear late in the course of the disease, delaying diagnosis.

Clinical presentation of persistent neurological symptoms prompts Magnetic Resonance Imaging (MRI) or X-ray computed tomography (CT), often followed by a stereotactic biopsy or craniotomy, with tumor resection and histological analysis, in order to confirm the pathology and help delineate the appropriate therapeutic response. Imaging of tumor blood flow using

perfusion MRI and measuring tumor metabolite concentration with magnetic resonance spectroscopy, may also add value to standard MRI and may be useful for differentiation of recurrent tumor from changes inherent to treatment.[13]

1.2.3 Conventional treatment

Despite the great progress in understanding GBM pathogenesis, current therapeutic approaches are still very limited and ineffective in stopping cancer progression.

Standard treatment for newly diagnosed patients consists in a combined therapy, often palliative, which includes surgical resection followed by radiotherapy and concomitant or adjuvant chemotherapy.[20,21]

Besides causing severe side effects, including toxicity to adjacent brain structure, as well as long-term effects such as cognitive deficits and epilepsy, this therapeutic approach provides an average patient survival of only 12 to 15 months. Surgical removal is compromised by the invasive nature of glioma cells, and always leaves a residual population of infiltrating cells that are responsible for tumor recurrence, as well as radiation and chemotherapy resistance. The complete removal of these cells, even if possible, would cause severe neurological deficits.[20]

Currently, the main chemotherapeutic agent used for GBM treatment is temozolomide (TMZ), an oral alkylating agent of the imidazotetrazine class, capable of penetrating the blood-brain barrier.[22]

TMZ is a small (194 Da) lipophilic molecule that acts as a prodrug: stable at acidic pH values, which enables oral administration, yet labile

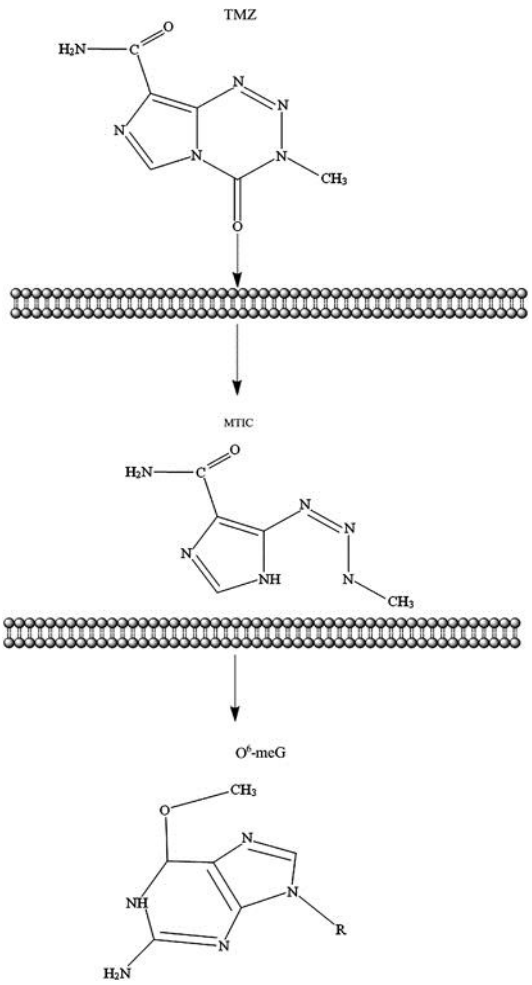

Figure 1- Mechanism of action of TMZ. TMZ underdoes spontaneous breakdown at physiological pH to form MTIC, a reactive intermediate that further reacts with water and liberates methyldiazonium cation, which methylates DNA at the O^6 and N^7 position of a guanine residue as well as N^3 position of an adenine residue.[98]

above pH 7. Thus, TMZ is rapidly absorbed intact, but then undergoes spontaneous breakdown to form monomethyl triazene 5-(3-methyltriazen-1-yl)-imidazole-4-carboxamide (MTIC). MTIC subsequently reacts with water to liberate 5-aminoimidazole-4-carboxamide (AIC) and the highly reactive methyldiazonium cation, which methylates DNA at the N^7 position of guanine rich regions, followed by methylation of N^3 adenine and O^6 guanine residues (Figure 1). [22–24]

TMZ activation occurs preferentially within tumor tissue, since it requires a narrow pH window, close to physiological pH, and brain tumors possess a more alkaline pH compared with the surrounding healthy tissue.[24]

The incapacity of the DNA mismatch repair system (MMR) to find a complementary base for methylated guanine appears to be responsible for the cytotoxic effects exerted by this drug. Unrepaired O^6- methylguanine (O^6-MeG) mispairs with thymine (rather than cytosine) during DNA replication, triggering the DNA mismatch repair (MMR) system, which recognizes exclusively the mispaired thymine on the daughter strand and excises it, leaving intact the O^6-MeG in the template strand. Therefore, futile cycles of thymine reinsertion and excision result in long-lived DNA nicks that accumulate and persist into the subsequent cell cycle, causing replication fork collapse, ultimately leading to G_2/M cell cycle arrest, and apoptosis.

However, direct repair of O^6-MeG by the suicide enzyme methylguanine-DNA methyltransferase (MGMT) frequently results in TMZ resistance. MGMT is a small protein (22 kDa) present in both cytoplasm and nucleus that reverts the cytotoxic effect of TMZ by transferring the O^6-MeG from a guanine residue to a cysteine residue in its active site (Cyst 145). This enzyme binds to the damaged base in the minor groove of DNA, causing the base to flip out of the helix and bind to its cysteine residue. This alters the conformation of the DNA binding domain allowing MGMT detachment and further degradation through the ubiquitin/proteosomal system. Furthermore, a deficiency in the MMR pathway results in a failure to recognize and repair the O^6-MeG adducts produced by TMZ and also render cells tolerant to the cytotoxic effects of this drug. DNA replication continues past the O^6-MeG adducts without cell cycle arrest or apoptosis[24,25].

It has become clear that the conventional treatment for patients with a malignant glioma suffers from several limitations. Therefore, advances in GBM therapy are urgently needed to improve the patients overall survival and reduce the side effects common to most anti-cancer medical treatments.

1.3 Cancer stem cells in GBM

1.3.1 Origin and common features

Over the past decade, our vision on the cellular origin of cancer and the mechanisms underlying its growth has slightly changed.[26] Currently, there are two models that explain tumor heterogeneity (Figure 2): 1) the stochastic model, in which each tumor cell is biologically equivalent and possess the ability to repopulate and regenerate the tumor itself, and in which any heterogeneity due to random intrinsic or extrinsic influences that alter the behavior of individual cells in the tumor, promoting their survival, aggressiveness and metastic potential; and 2) the hierarchy model, which states that tumors are composed of biologically distinct cell classes with differing functional properties and behavior. Among them, a subpopulation of tumor cells with distinct stem-like properties is thought to be responsible for tumor initiation and growth. These cancer stem cells (CSCs) can divide asymmetrically, originating an identical daughter cell and a more differentiated cell, which after subsequent divisions, gives rise to the bulk of the tumor.[27–29]

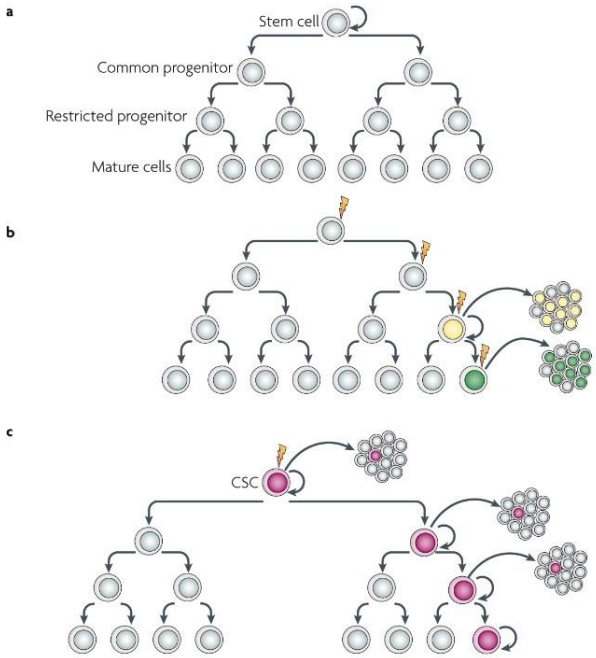

Figure 2- Models of tumor heterogeneity. (a) Normal cellular hierarchy, comprising a stem cell and the subsequent progenitor and mature cells. (b) Stochastic or clonal model, in which tumor cells are biologically equivalent and display similar tumorigenic potential. (c) Hierarchy model, hypothesizing that only a subset of cells with stem-like properties has the capacity to initiate tumor growth. [27]

The first prospective identification of a CSC was reported by Dick and colleagues in 1997 for human acute myeloid leukemia (AML), when they found that a rare malignant cell had the potential to initiate the original disease after transplantation into immunodeficient mice.[28,30] Six years later, in 2003, Al-Hajj *et al.*, identified and isolated for the first time CSCs from a solid tumor using specific markers.[31] Since then, driven by these author's work, CSCs have been identified in a variety of solid tumors, including GBM.[26]

It remains elusive whether CSCs arise from mature cells that acquired the ability to self-renew as a result of tumorigenic mutations or their normal stem cell counterparts. However, since differentiated cells have very limited lifespan, it is unlikely that the multiple mutations required for a cell to become malignant could occur during the short life of these cells. On the other hand, the longevity of normal stem cells makes them susceptible to accumulation of genetic and epigenetic mutations that could result in neoplastic transformation.[31,32] Furthermore, CSCs share many features with normal stem cells, including self-renew, multipotent differentiation, migration capacity as well as tightly regulated self-renewal pathways- Wnt-β-catenin, sonic hedgehog, Notch and PTEN signaling. In addition, CSCs appear to show increased antiapoptotic activity and drug resistance, due to increased expression levels of drug efflux pumps, affinity for hypoxic environments and also ability to remain quiescent for extended periods of time, which makes them resistance to standard antiproliferative treatments, ultimately facilitating tumor relapse (Figure 3).[26,27,31,33]

1.3.2 Identification and isolation

In order to develop new therapies aiming to target CSCs, it is imperative to functionally define and characterize them.[31]

Although the most reliable way to differentiate CSCs from the remaining tumor population is through their potential to give rise to tumors comprising both new CSCs and heterogeneous populations of differentiated cells, upon transplantation into immunocompromised mice, several other methodologies are currently employed to identify and isolate these residual population of stem-like cells: isolation by flow cytometry using CSCs specific cell surface markers such as CD133; detection of side-population phenotypes by Hoechst 33342 exclusion; ability to grow as floating spheres in serum-free medium and assessment of aldehyde dehydrogenase (ALDH) activity.[32]

Although different cell surface antigens have been identified in several cancers as possible CSCs markers, none of them are exclusively expressed by CSCs. Also, several studies support the evidence that the phenotype of these cells varies among different tissues and even within the same population, highlighting the need to find specific markers or use a combination of different markers for a more accurate isolation.

1.3.3 Glioblastoma Stem cells (GSCs)

In 2004, the identification of a $CD133^+$ cell subpopulation that exhibit stem-like properties both *in vitro* and *in vivo* opened new doors to a better understanding of GBM pathogenesis and established a new promising cellular target for more effective therapies.[34,35] In this study, Singh and colleagues demonstrated that a small population of only 100 $CD133^+$ cells, defined as glioblastoma stem-like cells (GSCs), were able to initiate tumors that were phenotypically identical to the patient's original tumor, upon transplantation into immunocompromised mice, whereas injection of 10,000 $CD133^-$ cells didn't demonstrate any tumorigenic potential.[34] Since then, whether relying on cell surface markers, culture conditions or functional properties, several groups have successfully isolated CSCs from primary glioblastomas.[35]

Currently, the most direct approach for GSCs enrichment is via magnetic sorting or flow cytometry sorting using CD133 expression as a GSC marker.[35]

CD133 (Prominin 1 or AC133) is a pentaspan transmembrane glycoprotein, originally identified as a surface antigen expressed on hematopoietic stem cells. Its function in normal and malignant tissues remains elusive, although recent studies suggest a role in the regulation of cancer cell proliferation and colony forming capacity.[36,26]

Despite cell sorting for CD133 expression can efficiently isolate cells with tumorigenic potential, the utility of this protein as a reliable marker for GSCs has been questioned in several studies. In fact, some authors demonstrated that $CD133^-$ glioma cells were also able to self-renew and regenerate tumors in xenotransplantation assays. In addition, this marker is not specific for GSCs since it has been detected on the surface of differentiated epithelial cells in a variety of tissues. Thus, different cell surface markers, such as CD44, CD15, A2B5 and alpha 6 integrin have recently been employed in GSCs isolation, alone or in combination with CD133.[36,26]

The ability of CSCs (and also normal NSCs) to propagate and expand indefinitely in serum-free medium supplemented with growth factors, such as epidermal growth factor (EGF) and basic fibroblast growth factor (FGF), in conditions in which most differentiated cells die, has also been explored as an alternative method for GSCs isolation.[34] However, recent studies, suggested an alternative culture method, based in the use of adherent cultures enriched with attachment factors, particularly laminin, which would result in a more uniform exposure to growth factors, oxygen and nutrients, leading in turn to a more homogeneous cell

population.[37,38] Flow cytometry sorting based on exclusion of Hoechst 33342 and sorting for ALDH1 activity also constitute different approaches that have been successfully employed in GSCs isolation.[36]

1.3.4 GSCs specific signaling pathways and therapeutic targets

GBM has one of the worst survival rates among all human cancers, and this scenario has been greatly attributed to the presence of GSCs within the tumor mass.[39]

Liu *et al*, reported for the first time that $CD133^+$ GSCs were significantly resistance to conventional chemotherapeutic agents, including TMZ, and this was correlated with the overexpression of drug resistance proteins such as BCRP1 (an ATP-binding cassette protein) and MGMT, as well as anti-apoptotic proteins, such as Bcl-2, FLIP, BCL-XL and the inhibitor of apoptosis protein cIAP1. In addition, $CD133^+$ cells also showed highly increased levels of CXCR4, a potent chemotactic agent, supporting the role of GSCs in GBM migration and invasion.[40]

Active tumor angiogenesis is another extensively studied hallmark of GBM.[39] Through the secretion of high levels of pro-angiogenic factors, such as VEGF and stromal-derived factor 1, GSCs promote the development of their own perivascular niche and neovascularization, which plays an essential role in providing nutrients and oxygen as well as removing waste in order to maintain the population of GSCs and sustain tumorigenesis. Numerous studies have shown that the degree of vascularization is closely correlated with tumor aggressiveness and poor clinical prognosis.[39,41,42]

Hypoxic and necrotic areas are common in GBM and also associated with an aggressive outcome. Hypoxia promotes self-renewal and prevents the differentiation of GSCs, as well as regulation of stem cell markers expression, including nestin, Sox2, Oct4, Nanog, c-Myc, Olig2 and Bmi1. These transcription factors are frequently overexpressed in GSCs and play a critical role in self-renewal, proliferation, differentiation and survival. Bmi1 is mostly involved in self-renewal, while oct4, sox2 and c-Myc are also required for cell survival.[41,42]

It is now clear that some of the signaling pathways and molecules involved in normal adult neurogenesis, have been altered and sequestered by GSCs, in order to support their tumorigenic potential. They include the Wnt, notch and sonic hedgehog-Gli pathways, as well as those mediated by the tyrosine kinase receptors (RTK), epidermal growth factor (EGF) and bone morphogenetic proteins (BMP), all of which appear to be involved in the regulation of

proliferation, differentiation, survival and drug resistance. For example, the Wnt pathway, which normally controls proliferation and self-renew of NSCs, is often overexpressed in GSCs, and its silencing results in a delay in tumor progression *in vivo*. Similarly, blocking of notch signaling attenuates neurosphere formation, proliferation and radioresistance, while inhibition of sonic hedgehog-Gli signaling pathway reduces self-renewal and migration, and sensitizes cells to TMZ. [43]

microRNAs have also been implicated in the maintenance of GSCs properties, playing critical roles in self-renewal, differentiation and fate determination.[44,45] Furthermore, several key proteins often dysregulated in GBM largely contribute to the pronounced malignancy of GSCs, in particular progranulin (PGRN). Bandey *et al*., recently reported that this protein, which is frequently overexpressed in GBM, renders cells resistant to TMZ and accentuates their tumorigenic potential by upregulating DNA repair proteins as well as cancer stemness genes, such as CD133 and CD44[46]. In addition, the DNA methyltransferases 3A and 3B, responsible for the maintenance of DNA methylation pattern as well as the *de novo* DNA methylation, are now implicated in suppressing the expression of differentiation and apoptosis- related genes in GSCs.[47]

Accordingly, all of the aforementioned signaling pathways might constitute attractive approaches for GSCs targeting.

1.4 microRNAs

MicroRNAs, are small, non-coding RNAs with an established role in cancer initiation and development.[48]

Recent studies discovered different miRNA signatures between normal cells and cancer cells, some of which are closely associated with cancer progression and prognosis, suggesting that gene therapy based on the modulation of miRNAs expression in tumor cells, might represent an attractive and effective therapeutic strategy for tumor eradication.[49,50]

1.4.1 MiRNAs biogenesis

MicroRNAs (miRNAs) are a class of evolutionally conserved non-coding RNAs, of approximately 12-25 nucleotides in length, that regulate gene expression through imperfect pairing between the seed region (nucleotides 2-8 at the 5' end) of the microRNA and the complementary sequence at the 3' untranslated region (3' UTR) of its mRNA targets, resulting in permanent/temporary translation suppression or direct degradation of target mRNA.[48,51,52]

MiRNAs are initially transcribed in the cell nucleus from intragenic or intergenic regions by RNA polymerase II to form long primary transcripts of 1-3 kb. These primary miRNAs are subsequently cleaved by the RNase III endonuclease Drosha and a double-stranded RNA-binding protein Pasha into long stem-loop precursor strands of approximately 70 nucleotides in length (pre-miRNAs). The pre-miRNAs are exported out of nucleus into the cytoplasm by Exportin-5, where they are bound to the Dicer. This type III RNase endonuclease, cleaves the pre-miRNA near the terminal loop, liberating a small RNA duplex, which is subsequently loaded onto one of the four known Argonaute proteins (AGO1-4), thus forming the RNA-induced silencing complex (RISC). Pre-RISC further removes the passenger strand of the small RNA duplex, generating a mature RISC. This process is dependent on the type of AGO, since

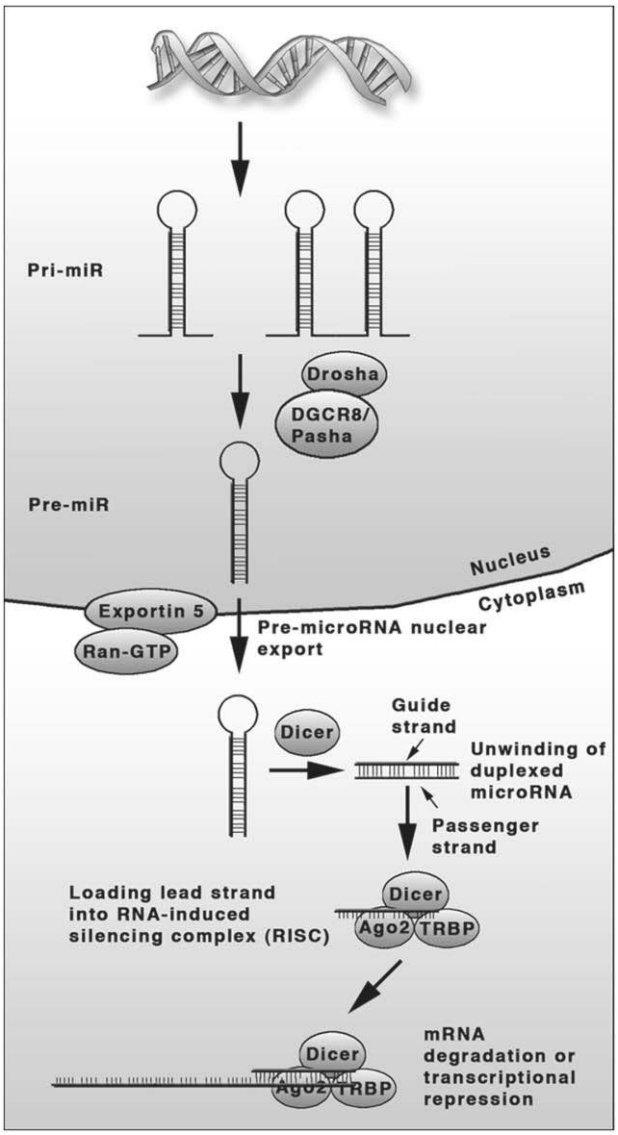

Figure 3- MicroRNA processing. Pri-miRNAs generated by RNA polymerase II are cleaved by endonuclease Drosha in the nucleus into pre-miRNAs that are subsequently exported to the cytoplasm and cleaved to mature miRNAs by endonuclease Dicer. RISC associates with miRNA and binds to complementary mRNA sequences, repressing their translation or inducing degradation.[61]

AGO 2 usually cleaves the passenger strand, whereas the remaining AGO proteins unwind the miRNA duplex, without cleavage (more frequent).[53] The mature strand recognizes complementary sequences in the 3' UTR of target mRNAs and guides the miRNA-RISC complex to repress gene expression (Figure 4). If the miRNA has complete complementary to its target mRNA, this will result in mRNA degradation, whereas when the pairing between miRNA and target is imperfect the outcome is cytoplasm sequestration of the mRNA from translational machinery.[48,51,54,55]

1.4.2 MiRNAs detection

It has been estimated that over 1,500 human miRNAs regulate approximately 30% of total genomic mRNA. Furthermore, each miRNA can target hundreds of genes and an individual mRNA can be coordinately suppressed by multiple miRNAs, thus these molecules play a pivotal role in the regulation of key cellular processes such as apoptosis, cellular proliferation, differentiation, development and metabolism.[54] Aberrant miRNA expression has been linked to initiation and development of several human diseases, including cancer. Therefore, it is essential to develop efficient and reliable strategies that allow detection of the expression levels of these molecules, in order to fully understand the role of miRNAs in the wide regulatory pathways underlying cancer malignancy.[51]

Although recently there has been an increase in the development of more specific and sensitive approaches, miRNA detection and analysis is still technically demanding, mostly due to their small size, low abundance and sequence similarity among family members, which presents a challenge to specific detection.

Currently, the more traditional approaches employed in miRNA profiling include northern blotting, microarrays and quantitative RT-PCR (qRT-PCR). Northern blotting is the most simple and widespread method to assess miRNA levels, since it does not require special equipment and allows both quantification of a given miRNA expression and size determination. Nevertheless, this technique depend upon a large amount of total RNA as starting material, and often fails to detect miRNAs with low abundance. MiRNA microarray is often used for profiling large numbers of microRNAs. However, since this method presents low sensitivity and dynamic range, it is often used as a screening tool rather than as a quantitative method, thereafter requiring further validation to quantify the miRNA expression levels. qRT-PCR has the widest dynamic range and accuracy, providing absolute quantification, with the only

disadvantage of requiring RNA isolation. For this reason, this method is considered the gold-standard for miRNA expression, and often used to validate results obtained by northern blotting and miRNA microarrays.[51,56]

1.4.3 MiRNAs in cancer

Since *Calin et al.,* described the deletion of miR-15 and miR-16 loci in the majority of samples from patients with B cell chronic lymphocytic leukemia (B-CLL), the interest in understanding how miRNAs regulate gene expression and their role in cancer development has been growing.[57]

Ever since, several publications reported both loss-of-function and gain-of-function experiments that seemed to firmly indicate that miRNAs play key roles in cancer initiation and progression.[58,59]

Although miRNAs can either have an oncogenic or tumor-suppressive function, studies provided evidence that miRNA expression is globally suppressed in tumor cells compared with normal tissue, suggesting that the miRNA biogenesis pathway might be impaired in cancer and this may be responsible for the increased tumor growth and metastasis.[55]

Similar to miR-15 and miR-16, a large number of miRNAs are located at fragile sites or within genomic regions that are deleted, amplified or translocated in cancer. These variations alter pre-miRNA transcription and miRNA expression, ultimately leading to aberrant expression of downstream target mRNAs that can promote cancer initiation and progression.

In addition to genomic mutations, miRNAs dysregulation can also arise from alterations in tumor suppressor or oncogenic factors that act as transcriptional activators or repressors of pre-miRNA.[55] Such is the case of the proto-oncoprotein MYC, which activates the expression of oncogenic miRNAs, including the miR-17~92 cluster. These miRNAs promote cancer progression by controlling the expression of thrombospondin 1 (THBS1), connective tissue growth factor (CTGF) and other target mRNAs that regulate cell cycle progression and angiogenesis.

On the other hand, expression of the miR-200 (miR-200a, miR-200b and miR-200c) family is frequently suppressed in several types of tumors. This family targets the mRNAs encoding the zinc-finger E-box-binding homeobox (ZEB) transcription factors, ZEB1 and ZEB2, which suppress the expression of epithelial genes that promote epithelial-mesenchymal transition (EMT).

Epigenetic modification of histone proteins and DNA is also a common feature of cancer pathogenesis that drives to dysregulation of miRNA expression. Indeed, the CpG islands at the gene promoters of tumor-suppressive miRNAs are frequently hypermethylated in cancer, thereby causing epigenetic silencing. Furthermore, histone modifications also promote chromatin remodeling and cooperate with DNA methylation to suppress miRNA expression.

miRNAs not only are regulated by DNA methylation but also modulate DNA methylation by interfering with the DNA methylation machinery.[48,55]

1.4.4 MiRNAs in GBM

Considerable progress has been made towards understanding the role of miRNAs in glioblastoma.[12] Since Ciafré *et al*., first described different miRNA expression signatures between GBM tumor samples and normal brain tissue, the number of publications on this subject has increased exponentially and now it is evident that an impairment in the miRNA regulatory network is one of the key mechanisms involved in GBM pathogenesis.[60]

Besides being involved in many processes that drive tumorigenesis, such as cell proliferation, cell cycle regulation, apoptosis, invasion and angiogenesis, miRNAs also play an important role in regulating GSCs behavior, which has been suggested as a mechanism for resistance to standard therapeutic approaches. Thus, regulation of aberrant miRNAs could improve not only conventional radio- and chemotherapy, but also the sensitivity of GBM to molecular target therapy.[61] Some of the miRNAs frequently deregulated in GBM are comprised in table 1.[54]

Table 1. Expression and function of several microRNAs dysregulated in GBM.

miRNA	Functions	Expression pattern in GBM
miR-7	Differentiation, invasion, proliferation, migration, radioresistance, apoptosis	Down
miR-9	Proliferation, self-renewal	Up
miR-10a/miR-10b	Apoptosis, autophagy, chemoresistance, invasion, prognosis, proliferation, senescence, tumor growth	Up
miR-17-92	Cell cycle, proliferation	Up
miR-18a	Angiogenesis, growth, apoptosis, proliferation	Up
miR-20a	Angiogenesis, growth, proliferation	Up
miR-21	Apoptosis, chemoresistance, invasion, proliferation, tumor growth	Up
miR-26a/miR-26b	Apoptosis, proliferation, tumor growth, vasculogenic mimicry	Up
miR-29b	Apoptosis, invasion, Proliferation, DNA methylation	Down
miR-30e	Proliferation, invasion, angiogenesis, tumor growth	Up
miR-32	Proliferation	Down
miR-34a	Apoptosis, cell cycle, differentiation, invasion, proliferation, tumor growth	Down
miR-92	Proliferation, apoptosis	Up
miR-93	Angiogenesis, tumor growth, proliferation	Up
miR-101	Invasion, angiogenesis, proliferation, tumor growth, migration, apoptosis	Down
miR-124a	Cell cycle, differentiation, invasion, proliferation	Down
miR-125a/miR-125b	Apoptosis, invasion, proliferation	Down
miR-128	Proliferation, self-renewal, tumor growth	Down
miR-137	Cell cycle, differentiation, proliferation	Down
miR-146b-5p	Invasion, migration	Down
miR-153	Apoptosis, proliferation	Down
miR-181a/miR-181b/miR- 181c	Apoptosis, colony formation, invasion, proliferation, radiosensitivity	Down
miR-218	Migration	Down
miR-221/miR-222	Apoptosis, cell cycle, CTL-mediated tumor lysis, invasion, proliferation	Up
miR-296	Angiogenesis	Up
miR-302-367 cluster	Differentiation, invasion, proliferation, selfrenewal, stemness	Up
miR-326	Apoptosis, invasion, metabolism, proliferation, tumor growth	Down
miR-381	Proliferation	Up
miR-451	Apoptosis, cell cycle, chemoresistance, invasion, proliferation, proliferation/migration regulation	Up

1.4.4.1 MiR-29b family

One of the many miRNA families with a validated role in GBM that is currently under heavy investigation is the miR-29 family. Downregulation of miR-29 members, in particular miR-29b, has been reported not only in GBM but also in a variety of human cancers, and is often associated with a poor prognosis.[62]

The miR-29 family consists of three mature members, miR-29a, miR-29b and miR-29c, that share the same "seed region" (nucleotides 2-7), and hence overlap in their predicted mRNA targets. The genes encoding miR-29 members are organized in two clusters, miR-29a/b-1 and miR-29b-2/c, which are transcribed together as a polycistronic primary transcript, and are located on human chromosomes 7q32.3 and 1q32.2, respectively. Despite similar sequences, this family presents different subcellular compartmentalization, since miR-29c is primarily localized in the cytoplasm, while miR-29a and miR-29b are concentrated in the nucleus. Mature miR-29 sequences are highly conserved between humans and mice and ubiquitously expressed at the organ level in both species, with highest expression in the brain and heart. [63]

Based on sequence prediction, miR-29 family members have up to 7000 predicted targets. However, so far, few have been experimentally validated.

According to several authors, this family has been implicated in a wide range of cellular processes, including regulation of extracellular matrix, cell proliferation, differentiation and apoptosis. Kriegel *et al*. reported that all three miR-29b family members target at least 16 genes that code for several key proteins involved in the physiological or pathological formation of extracellular matrix, including collagen isoforms, laminin γ1, elastin, matrix metalloproteinase 2, among others. Loss of regulation of extracellular matrix or related proteins, due to miR-29 downregulation, may contribute to cell migration in GBM. MiR-29 family members can also act as tumor suppressors, by targeting p85α and CDC42, thus relieving the suppression of p53, a transcription factor responsible for controlling the expression of genes that regulate cell growth, senescence, apoptosis and genome integrity. In addition, they target several oncogenes and pro-apoptotic proteins, such as Tcl-1, an Akt kinase coactivator with a central role in regulating intracellular survival, the Bcl-2 family member Mcl-1 and also CDK6, an oncogene required for cell cycle progression into S-phase.[64]

In silico studies predict a binding site for miR-29b, the main target of this work, in the 3'UTR of several proteins with known functions in GBM tumorigenesis, in particular the *de novo* DNA methyltransferases, DNMT3A and DNMT3B, the transcription factor YY1 and

progranulin, which regulate two major events on cancer development: epigenetic modifications and invasion and metastasis, respectively.[65,66]

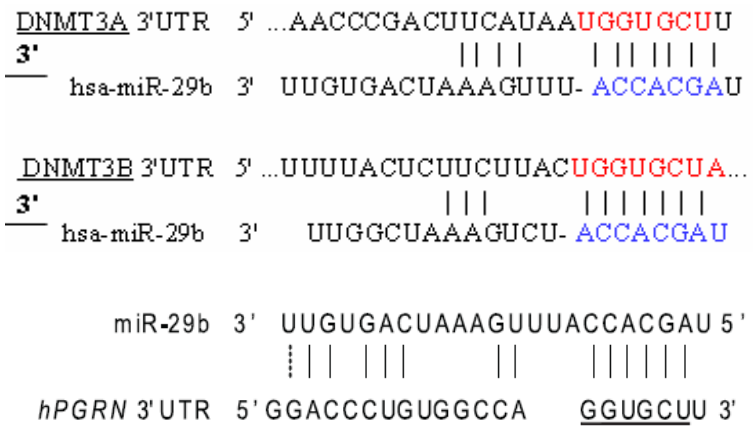

Figure 4 – PGRN and DNA methyltransferases 3A and 3B are validated targets of miR-29b. Schematic representation of miR-29b binding site in the 3'UTR of PGRN, DNMT3A and DNMT3B mRNAs.[65,89]

1.5 Progranulin

1.5.1 PGRN structure

Encoded by the *GRN* gene on chromosome 17q21, PGRN (granulin-epithelin precursor, PC-cell derived growth factor or acrogranin) is a secreted glycoprotein of approximately 88 kDa, composed of seven and a half tandem repeats of a 12-cysteine motif, called the granulin domains (Figure 5).

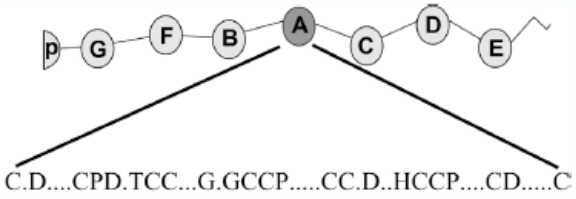

Figure 5 - Schematic representation of granulin domains, and their 12-cystein motif.[69]

The cysteine motifs form six disulfide bridges and the peptide backbone adopts a three-dimensional structure of a parallel stack of beta-hairpins in the form of a left handed helix. The proteolytic cleavage of PGRN by extracellular proteases gives rise to smaller peptides called granulins (GRNs) or epithelins, which range in size from 6 to 25 kDa and are involved in many biological functions, acting mainly as inflammatory mediators.[67] PGRN is ubiquitously expressed in immunological tissues, such as spleen and lymph nodes, as well as epithelial cells,

particularly those with a rapid turnover, such as skin and gastrointestinal tract cells, hematopoietic cells, neurons and microglia. [67,68,69]

1.5.2 Biological roles

Wang *et al*. reported for the first time, that PGRN expression was upregulated in astrocytomas and this was positively correlated with pathological grading and closely associated with poor prognosis, suggesting not only that this growth factor might play a crucial role in malignant progression and aggressiveness, but also serve as a prognostic biomarker for GBM.[70] Supporting this hypothesis, several studies showed that while overexpression of PGRN promotes tumorigenesis *in vivo*, its depletion by mRNA targeting inhibits xenograft tumor growth.[68]

In addition to its normal regulatory role in wound repair, cellular mitosis and inflammation, PGRN appears to regulate and support several biological processes that often drive tumorigenesis, including enhanced cell proliferation, survival, migration, invasion and angiogenesis[68,70,71], all of which are known to be associated with activation of growth factor-related signaling events, including PI3k/AKT (phosphatidylinositol 3'-kinase), MAPK/ERK (mitogen-activated protein kinase) and FAK (focal adhesion kinase) pathways.[71]

In fact, an increase in phosphorylation of several signaling molecules in the FAK signaling pathway, particularly endophilin A2, appears to increase the expression of the matrix-degrading metalloproteinases MMP2, MMP9, MMP13 and MMP17, which promote degradation of components of the extracellular matrix and allow subsequent cell detachment and migration[68].

Cellular invasion is supported by the PGRN-induced resistance to apoptosis not only caused by chemotherapeutic agents, such as TMZ[46], but mainly by *anoikis*, a form of programmed cell death that occurs when anchorage-dependent cells detach from the surrounding extracellular matrix.[69]

Considering the diversity of effects that a deregulation of PGRN expression can have on tumor development and progression, particularly in cell motility and resistance to chemotherapeutic agents, therapeutic strategies that target this protein may significantly improve the efficacy of current regimen for GBM, as well patient's outcome.

1.6 DNA methylation

Currently it is accepted that cancer initiation and progression is not only controlled by genetic modifications but also by epigenetic events, which can be defined as mitotically heritable changes in gene expression that are independent of alterations in the primary DNA sequence.[72,73] Epigenetic mechanisms are reversible and include DNA methylation, histone modifications and posttranscriptional gene regulation by miRNAs. Since these mechanisms play a crucial role in development and growth, any disruption can lead to altered gene expression and malignant cellular transformation.[72]

In mammals, DNA methylation is catalyzed by DNA methyltransferases (DNMTs), including DNMT1, DNMT3A and DNMT3B, and consists in addition of a methyl group to a cytosine residue to create 5-methylcytosine, normally at the 5´ position of a cytosine ring within CpG dinucleotides, often resulting in gene silencing.[72] DNMT1 is responsible for replicating the DNA methylation pattern in genomic DNA, whereas DNMT3A and DNMT3B account for the *de novo* DNA methylation, targeting unmethylated CpG dinucleotides. These enzymes are frequently referred to as *de novo* DNA methyltransferases and are highly expressed during embryogenesis and less expressed in adult tissues. [72]

The CpG dinucleotides are concentrated in dense pockets called CpG islands, where 50-60% of all gene promoters lie.

The establishment and maintenance of DNA methylation is critical for developmental processes, as well as for genomic integrity, imprinting and transcriptional regulation.[72]

Post-translational modification of histone N-terminals (by acetylation, methylation, phosphorylation, ubiquitination, and others) constitute another well-established mechanism of epigenetic modification that can result in chromatin remodeling with consequent activation or repression of gene expression. This reversible process is controlled by a group of enzymes that include histone acetyltransferases (HATs), deacetylases (HDACs), methyltransferases (HMTs) and demethylases (HDMs), among others.[73,74]

The first association between DNA methylation and cancer was made in 1983, based on a study that showed a clear pattern of hypomethylation in cancer cells compared to their normal counterparts. Currently it is known that the majority of cancer cells show genome-wide hypomethylation, commonly in repetitive regions of the genome and frequently resulting in genomic instability and aberrant expression of oncogenes, as well as locus-specific

hypermethylation, mostly at CpG island promoters of genes involved in tumorigenesis and tumor progression.[72]

Recent studies show that global hypomethylation occurs in approximately 80% of all primary glioblastomas. The extent of hypomethylation may vary between individual tumors, with higher grades being associated with demethylation and transcriptional activation of the oncogene MAGEA1 as well as increased cellular proliferation. Despite very common, the molecular mechanisms inherent to GBM hypomethylation are still poorly understood.[73]

Locus-specific hypermethylation is also frequent in GBM, and usually affects genes with key roles in cell cycle control (CDKN2/p16, $p14^{arf}$, RB1 and phosphatase and tensin homolog gene, PTEN), DNA repair, apoptosis (TP53, bcl-2, bax, XAF-1), angiogenesis, invasion (protocadherin-gamma subfamily A11, PCDH-gamma-A11) and drug resistance (MGMT, suppressor of cytokine signaling 1, SOCSI).[73,75,76]

Aberrant histone modifications were also reported in GBM, and have been associated with a predisposition of tumor suppressor genes to DNA hypermethylation. Furthermore, some studies reported a deregulation of genes involved in the control of histone modifications, particularly, BMI-1, a member of the polycomb group complex that regulates histone H3K27 methylation.[73,74]

Recent studies support the evidence that the *de novo* DNA methyltransferases, DNMT3A and DNMT3B, are overexpressed in malignant gliomas and are, therefore, responsible for the hypermethylation pattern of tumor suppressor genes observed in the majority of cases, as well as for a deregulation of various signaling pathways detrimental to tumor initiation and progression.[77]

1.7 Targeted cancer therapy

Modulation of miRNA expression, either through reintroduction of a depleted miRNA, or inhibition of an overexpressed miRNA, has demonstrated a marked potential for application in GBM- targeted therapies, *per se* or in combination with radio- and chemotherapy.[12]

Nevertheless, the delivery of miRNAs *in vivo* presents several major limitations that include rapid degradation in serum conditions, lack of reliable delivery to the intracellular space and also delivery across the blood brain barrier (BBB). Thus, it is imperative to design delivery vehicles effective in overcoming cellular and physiological barriers, serum opsonization and

possible degradation by blood nucleases, and that are, simultaneously, target specific, in order to improve bioavailability and minimize side effects.[78]

To date, several formulations and molecular carriers have been proposed. Strategies to inhibit overexpressed miRNAs frequently include small molecule inhibitors that regulate microRNA processing, antisense oligonucleotides, locked nucleic acid constructs (LNA) and also antagomiRNAs. On the other hand, in order to increase the levels of downregulated miRNAs, researchers commonly opt for miRNA mimics or adeno-associated viruses. The use of liposomes, polymers, hydrogels and nanoparticles has been under investigation. However, so far, few trials have reached beyond phase I, suggesting that much remains to be made. [61,79]

Recently, a new class of lipid-based nanoparticles, designated stable nucleic acid lipid particles (SNALPs) were very effective in delivering small interfering RNAs (siRNAs), both *in vitro* and *in vivo*.[80] These particles, of approximately 120 nanometers in diameter, are formed by a lipid bilayer of cationic and fusogenic lipids coated with diffusible polyethylene glycol, and are characterized by high vesicle loading, good transfection efficiency and high stability in serum. Moreover, they can be modified in order to covalently bind to targeting peptides, enabling tumor-specific delivery, while reducing side effects. An example of this type of targeting ligand is chlorotoxin (CTX), a scorpion-derived peptide capable of binding to matrix metalloproteinase-2, an enzyme specifically overexpressed in glioma cells and related cancers, but poorly expressed in brain and normal tissues.

Our group recently succeeded in delivering anti-miR-21 oligonucleotides to GBM cells using CTX-coupled SNALPS as a vehicle[81], thus suggesting this system might also represent an attractive approach for the efficient delivery of miR-29b to GBM cells.

CHAPTER 2 - Objectives

2.1 Objectives

The major goal of this work is to study the role of miRNA-29b in the regulation of key processes that drive GBM progression and malignancy, and ultimately assess the therapeutic potential of miR-29b overexpression, per se, or in combination with chemotherapeutic agents currently involved in GBM treatment.

Accordingly, the main objectives of this study were:

- Isolate $CD133^+$ GSCs from both a primary and a recurrent human glioblastoma cell lines, U87MG and DBTRG-05MG respectively, using magnetic-activated cell sorting (MACS);

- Characterize and validate $CD133^+$ cells as GSCs through the evaluation of their ability to form neurospheres and their expression of stemness genes;

- Assess and compare miRNA-29b expression in $CD133^+$ cells, $CD133^-$ cells and human astrocytes;

- Modulate miR-29b expression in both U87MG and DBTRG-05 cell lines through the delivery of a synthetic miR-29b mimic;

- Quantify miR-29b and miR-29b target expression following cell transfection;

- Evaluate global DNA methylation status following miR-29b modulation;

- Investigate possible synergistic effects of a combined therapeutic strategy involving miR-29b modulation and cell treatment with sunitinib, axitinib or temozolomide.

CHAPTER 3 - Material and Methods

3.1 Cell lines and culturing conditions

The U87MG human glioma cell line was maintained in DMEM (Dulbecco's Modified Eagle's Medium) (Sigma-Aldrich, D5648), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Paisley, Scotland), 100U/mL penicillin (Sigma-Aldrich), 100 µg/mL streptomycin (Sigma-Aldrich),10mM HEPES and 12mM sodium bicarbonate. The DBRTG-05MG human recurrent glioma cell line was maintained in RPMI-1640 (Sigma-Aldrich, R4130), supplemented with 10% heat-inactivated FBS (Gibco, Paisley, Scotland), 100 U/mL penicillin (Sigma-Aldrich), 100µg/mL streptomycin (Sigma-Aldrich) and 12mM sodium bicarbonate. Both cell lines were cultured in adherent conditions at 37ºC under a humidified atmosphere containing 5% CO_2. The $CD133^+$ subpopulation isolated from both U87MG and DBTRG-05MG cell lines was maintained in serum-free DMEM-F12 (Sigma-Aldrich, 32500) supplemented with 20µg/mL B27 and 0.02µg/mL bFGF/EGF, and cultured in sphere-forming conditions at 37ºC under a humidified atmosphere containing 5% CO_2.

3.2 Isolation of CD133+ subpopulations by MACS (Magnetic-Activated Cell Sorting)

Magnetic cell sorting (MACS System, Miltenyi Biotec) was used to isolate the $CD133^+$ subpopulations from both U87MG and DBTRG-05MG cell lines. Cells were firstly dissociated and centrifuged at 1200 rpm for 5 minutes. The pellet was then resupended in PBS containing 0.5% bovine serum albumin (BSA) and 2mmol/L EDTA (MACS buffer) (at a concentration of 30µl/10^7cells), Fcr blocking reagent (Miltenyi Biotech, Bergisch-Gladbach, Germany) and anti-CD133 antibodies conjugated with magnetic beads (#130-050-801, Miltenyi Biotech, Bergisch-Gladbach, Germany) (both at a concentration of 10µl/10^7 cells), and incubated with rotation at 4ºC for 15 minutes. After incubation, cells were once again centrifuged at 1200 rpm for 5 minutes, resupended in MACS buffer and sorted by a magnetic column following the manufacturer's instructions (Miltenyi Biotech, Bergisch-Gladbach, Germany). Following separation, the $CD133^+$ subpopulations were eluted with MACS buffer, upon column removal from the magnetic field. Both $CD133^+$ and $CD133^-$ sorted fractions were cultured for 4 days for number and marker enrichment. The $CD133^+$ cells were maintained in serum-free DMEM-F12 supplemented with 20µg/mL B27 and 0.02µg/mL bFGF/EGF, under sphere-forming conditions

(low-adhesion plates), while the $CD133^-$ cells were maintained in DMEM/RPMI under adherent conditions. $CD133^-$ cells were also occasionally cultured in the same conditions as the $CD133^+$ subpopulation, for cell image acquisition.

3.3 Liposome preparation and cell transfection

The miRIDIAN miR-29b mimic and the double-stranded oligonucleotide control (Dharmacon) were resupended in sterile water and stored in 20μM aliquots at -20ºC.

For cell transfection, two transfection reagents were employed: DLS liposomes and DharmaFECT Duo (Dharmacon). DLS (delivery liposomal system) stock solution was prepared by mixing 1 mg of dioctadecylamidoglycylspermidine (DOGS, Promega) and 1 mg of dioleoyl phosphatidylethanolamine (DOPE, Sigma-Aldrich) in 40μL of 90% ethanol, followed by addition of 360μL of sterile H_2O. The mixture was homogenized by vortex and incubated for 30 min to allow liposome formation.[82]

Both U87MG and DBTRG-05MG cell lines were plated into 48-well plates (Costar), with a density of $2x10^4$ cells/well and a final volume of 300μL of culture medium (DMEM and RPMI, respectively). On the following day, a mixture of sterile HBS, DLS and miR-29b oligonucleotide or scrambled oligonucleotide was prepared. To achieve a final concentration of 50nM of oligonucleotide/well (miR-29b or scrambled control), the mixture contained 0.5μL of oligonucleotide (10pmol), 1.06μL of DLS (considering a ratio of 190 μg DLS/ 10 μg oligonucleotide) and 8.44μL of sterile HBS per well. After 30 min of incubation at room temperature 10 μL of the mixture was added to 190 μL of serum-free OPTIMEM, in order to make up for a final transfection volume of 200 μL per well. After 4h of incubation at 37ºC, the transfection was stopped by replacing the OPTIMEM medium with the corresponding culture medium.

For cell transfection with DharmaFECT Duo, two separate mixtures were prepared: one containing 1μL of DharmaFECT diluted in 9μL of OPTIMEM per well; and another containing 10pmol (1.5μL) of oligonucleotide (or 30pmol in the case of 12-well plates) diluted in OPTIMEM at a final volume of 10μL. After a 30 min incubation at room temperature, both contents were mixed for 5 min and 20μL of the mixture were added to 180μL (or 580μL in the case of 12-well plates) of OPTIMEM. The final concentration of oligonucleotides was 50nM per well. After 4h of incubation at 37ºC in a CO_2 incubator, OPTIMEM was replaced by the corresponding culture medium.

Total RNA extraction for quantification of miR-29b and target mRNAs was conducted 48h post-transfection in 12-well plates (with a density of $1x10^5$ cells/well).

3.4 Drug storage and treatment

Sunitinib malate and axitinib were kindly offered by Pfizer (Basel, Switzerland) and temozolomide was acquired from Selleckchem. Stock solutions of sunitinib 25mM, axitinib 10mM and temozolomide 150mM were prepared in DMSO (Sigma, Germany) and stored at -20ºC.

Twenty-four hours after transfection, both U87MG and DBTRG-05MG cells were incubated with each of the 3 mentioned drugs, at 37º,C under a humidified atmosphere containing 5% CO_2. After 48h of incubation, cell viability was assessed through the AlamarBlue assay and cell density was determined by the sulforhodamine B assay.

3.5 Evaluation of cell viability

Cell viability was assessed by AlamarBlue® assay 24h post-transfection and 48h after drug treatment. Briefly, cells were incubated at 37ºC with culture medium containing 10% (v/v) of Resazurin dye (Sigma-Aldrich), until reduction of Resazurin by viable cells to resorufin and consequent development of a pink coloration. One hundred and fifty microliters of medium collected from each well were transferred into a 96-well plate, and the absorbance was measured at 570nm (reduced form) and 600nm (oxidized form) in a microplate reader (SpectraMax Plus 384, Molecular Devices). Cell viability was calculated as a percentage with respect to non-treated control cells, according to the equation:

Cell viability (% of control) = ($\Delta Abs_{\text{treated cells}}$ - $\Delta Abs_{\text{negative control}}$) x 100 / ($\Delta Abs_{\text{positive control}}$ - $\Delta Abs_{\text{negative control}}$), $\Delta Abs = Abs_{570nm} - Abs_{600nm}$

3.6 Cell density determination

Cell density was evaluated by the sulforhodamine B colorimetric assay. Shortly, cells in each well were fixed with 250µL of a solution of 1% acetic acid in methanol, and stored up to 1 week at -80ºC. After fixation, the solution was aspirated, the plate was dried for 20 min at 37ºC and further incubated for 1h at 37ºC with 250µL of a new solution containing 0.5% SRB

and 1% acetic acid in water. The excess of SRB was washed 3 times with 1% acetic acid in water, and the plate was dried for 20 min at 37ºC. Cells were then incubated with 1mL of EDTA 10mM (pH=10) for 15 min under gentle agitation. Two hundred microliters of medium were transferred to a 96-well plate and the absorbance was measured at 540nm in a microplate reader (SpectraMax Plus 384, Molecular Devices).

3.7 RNA extraction and cDNA synthesis

Total RNA was extracted from U87MG and DBTRG-05MG cells using the miRCURY Isolation Kit (Exiqon), according to manufacturer's instructions for cultured cells. Briefly, cells were lysed with a solution containing 10µl of β-mercaptoethanol/mL of lysis buffer, total RNA was bound to a silica matrix provided by the manufacturer and treated with On column DNase I digestion set (Sigma-Aldrich), for maximum removal of residual DNA. After being washed, total RNA was eluted with 30µl of elution buffer, and quantified using the NanoDrop 2000 Spectrophotometer (Thermo Scientific). cDNA synthesis for miRNA quantification was carried out using the Universal cDNA Synthesis Kit (Exiqon). cDNA was produced from 10ng of total RNA in a 10µl reaction, according to the following protocol: 60 min at 42ºC followed by heat-inactivation of reverse transcriptase for 5 min at 95ºC. The newly synthetized cDNA was further diluted 1:40 with RNase-free water and stored at -20ºC. For mRNA quantification, cDNA synthesis was performed from 0.5µg of starting RNA, using the NZY First-Strand cDNA Synthesis Kit (NZYtech, Lisbon, Portugal), and after applying the following protocol: 10 min at 25ºC, 30 min at 50ºC and 5 min at 85ºC. In order to degrade the RNA template in cDNA: RNA hybrids, samples were incubated for 20 min at 37ºC with NZY RNase H (*E.coli*). The resulting cDNA was further diluted 1:20 with RNase-free water and stored at -20ºC.

3.8 Quantitative Real-Time PCR (qPCR)

All qPCR reactions were carried out in a StepOnePlus thermocycler (Applied Biosystems) using 96-well microtitre plates. MiR-29b expression was assessed using the SYBR Green Master Mix (Exiqon) and primers pre-design by Exiqon. Normalization was performed with Snord 44. A master mix was prepared for each primer set consisting of 5µL of SYBR Green and 1µL of primer. For each reaction, performed in duplicate, 6µL of master mix were added to 4µL of template cDNA. PCR cycle conditions consisted in polymerase activation and

DNA denaturation for 10 min at 95ºC and 45 amplification cycles at 95ºC for 10 s (denaturation) and 60ºC for 1 min at ramp-rate of 1.6ºC/s (annealing and elongation).

The expression of individual genes was quantified with the SsoAdvanced™SYBR®Green Supermix (Bio-Rad) and using specific primer sequences designed with Beacon Designer™ software and idtDNA primer design tools. mRNA levels were normalized to HPRT expression. Both the amount of cDNA and master mix added to the 96-well PCR plate was similar to miRNA PCR. The reaction conditions consisted in polymerase activation and DNA denaturation at 95ºC for 30 s, followed by 45 cycles at 95ºC for 10s (denaturation), 30s at 55-60ºC (dependent of primer annealing temperature), and 30s 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 10s, with a 0,5ºC increase at each step. The No Template Control (NTC) and the No Reverse Transcriptase Control (noRT) were assayed for each primer, in every experiment performed.

Fold changes of miRNA and mRNA levels were calculated according to the Pfaffl method using the levels of Snord 44 and HPRT as internal controls respectively, and taking into consideration the different amplification efficiencies of all genes and miRNAs analyzed. 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.

3.9 DNA extraction

Genomic DNA (gDNA) was extracted using the NZY Tissue gDNA Isolation kit (nzytech) according to the manufacturer's recommendations. Briefly, after cells were lysed with a lysis solution containing Proteinase K and SDS, the total DNA was absorbed in the NZYSpin Tissue Column, washed with recommended buffers and further eluted with sterile water. The quality and quantity of isolated gDNA was determined using a Nanodrop 2000 Spectrophotometer (Thermo Scientific).

3.10 Detection of global DNA methylation levels

In order to evaluate the extent of global DNA methylation in both U87MG and DBTRG-05MG cell lines the following protocol was applied: Firstly, cells were plated into 12-well plates at a cell density of $1x10^5$ cells per well. 12 hours after plating, cells were transfected, in

duplicate, either with miR-29b mimic or a scrambled oligonucleotide and 48h after cells were collected and genomic DNA was extracted.

The effect of miR-29b modulation on global DNA methylation status was assessed by measuring the level of 5-methylcytosine (5-mC) with the 5-mC DNA ELISA Kit (Zymo Research Corp., Irvine, CA, USA), following the instructions from the manufacturer. This kit relies on the recognition of 5-mC by a highly sensitive and specific anti-5-methylcytosine monoclonal antibody. Briefly, 100 ng of DNA samples (transfected cells and non-transfected control cells) along with the standard controls provided by the manufacturer were denatured and used to coat the ELISA plate with 5-mC coating buffer. After 1h of incubation at 37ºC, the plate was blocked with 5-mC ELISA buffer and a mixture of primary and secondary antibodies were added to each well and incubated overnight at 37ºC. On the following day, the plate was washed with 5-mC ELISA buffer and incubated 1h with HRP Developer. The absorbance was measured at 405 and 450 nm using a microplate reader (SpectraMax Plus 384, Molecular Devices). The result was expressed in percentage of 5-mC, which was calculated through the second-order regression equation of the standard curve generated with controls of known 5-mC percentage.

3.11 Statistical analysis

Data are presented as mean ± standard deviation (SD) of at least three independent experiments, each performed in duplicate, unless stated otherwise. One way ANOVA combined with the Tukey posthoc test was used for multiple comparisons and considered significant when $p < 0.05$. Statistical differences are presented at probability levels of $p < 0.05$, 0.01 and 0.001. Calculations were performed with Prism 6 (GraphPad, San Diego, CA, USA).

Chapter 4 - Results and Discussion

4.1 Isolation and characterization of Glioblastoma Stem Cells (GSCs)

Formulated for the first time over 40 years ago, the cancer stem cell hypothesis is currently the theory that better explains the origin and malignant hallmarks of GBM, including the acquired resistance to standard radio- and chemotherapy that often results in tumor recurrence.[83] According to this theory, tumors are sustained by a small subpopulation of cells that display stem-like properties such as ability to self-renew and differentiate into different cell types that constitute the bulk of tumor, thus making them a highly promising target for new therapeutic approaches that aim tumor eradication.[84,85] However, a better understanding of the biology of these cells as well as the development of new methods for their isolation and *in vitro* propagation are two major issues that still remain to be addressed.

With the purpose of characterizing and exploring miR-29b expression profile of GSCs, we began our work with the isolation of this subpopulation from both a primary and a recurrent human glioblastoma cell lines, U87MG and DBTRG-05MG, respectively, using magnetic-activated cell sorting (MACS) (Figure 6). GSCs were separated from the remaining population based on their expression of CD133, a cell surface protein that despite being recently questioned regarding its specificity, is still the marker most frequently employed in GSCs isolation.

Magnetic sorting of $CD133^+$ subpopulations presented some challenges. In fact, isolation of DBTRG $CD133^+$ cells frequently resulted in column blocking, which was attributed to their larger size when compared to U87 cells. Nevertheless, the post-isolation yield was almost four times higher than the one obtained for U87 cell sorting, which was consistent with the higher profile of malignancy displayed by this recurrent cell line (Figure 7).

Figure 6 - Isolation of Glioblastoma Stem Cells from U87MG and DBRTG-05MG human GBM cell lines by MACS. After incubation with magnetic beads conjugated with anti-CD133 antibodies, the cell suspension is loaded onto a MACS® Column, placed in a magnetic field. While $CD133^+$ cells are retained within the column, the unlabeled cells run through, allowing cell sorting. $CD133^+$ cells can be eluted after removing the column from the magnetic field.

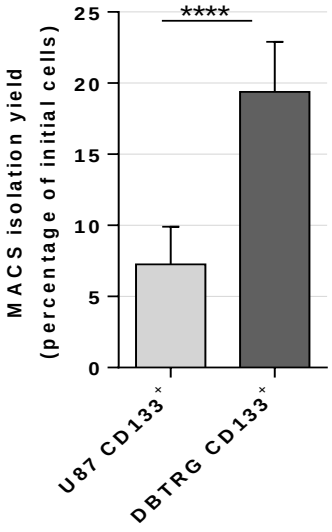

Figure 7 – Isolation of Glioblastoma Stem Cells from U87MG and DBTRG-05MG human GBM cell lines. Percentage of $CD133^+$ cells obtained from both U87 and DBTRG cell lines, after magnetic cell sorting. **** $p < 0.0001$. Values are presented as means ± SD.

Although $CD133^+$ cells have, undoubtedly, the unique capacity for self-renewal, as well as ability to initiate and drive tumor progression, recent studies support the evidence that $CD133^-$ cells also feature tumor-initiating activity, thus questioning CD133 as a reliable marker for GSCs isolation.[36] Therefore, in order to validate both U87 and DBTRG $CD133^+$ cells as GSCs we evaluated their ability to grow as floating spheres, a major hallmark of CSCs, as well as their expression of stemness genes.

Immediately after magnetic separation, both $CD133^+$ and a fraction of $CD133^-$ sorted cells were cultured for 4 days in serum-free DMEM-F12 supplemented with growth factors and under low adherent conditions, for number and marker enrichment, while the remaining $CD133^-$ subpopulations were maintained under adherent conditions in their corresponding culture medium.

According to the obtained results, and as previously described by Singh *et al.*[34], $CD133^+$ cells isolated from both cell lines had the ability to grow in suspension, forming colonies of neurospheres, and thus displaying self-renewal capacity (Figure 8). On the other hand, $CD133^-$ cells also had the ability to form neurospheres when cultured in the same conditions as the positive fraction, contrary to what would be expected since these cells should represent the fraction of differentiated cells that constitute the bulk of the tumor, and as a result shouldn't be able to survive or expand under sphere-forming conditions. These results might therefore be attributed to the presence of partially differentiated GSCs within the $CD133^-$ sorted fraction that do not express CD133 but remain pluripotent. Furthermore, dedifferentiation of $CD133^-$ cells during culture or unspecific sorting, resulting in the presence of $CD133^+$ cells in the negative fraction, might also constitute possible explanations for the obtained results.[36]

Besides their distinctive *in vivo* and *in vitro* behavior, GSCs can also be characterized by their gene expression profiles, in particular expression of stemness genes essential for maintaining stem cells in a pluripotent state.[38] Hence, in order to complement the results obtained in the neurosphere-forming assay, we assessed by qRT-PCR the expression levels of CD133, Nestin, CD15 and Bmi1, specific GSCs markers currently employed in their isolation, as well as the stemness markers Oct-4, Sox2 and Nanog, and the astrocytic and neuronal differentiation markers GFAP and Tuj-1, respectively.

Albeit both U87 and DBTRG $CD133^+$ fractions had the ability to grow as neurospheres under serum-free conditions, their stem phenotypes differed widely.

With regard to the U87 cell line, the results obtained were unpredictable, since $CD133^+$ cells expressed less CD133 marker than the negative fraction, as well as the stem-cell markers Oct-4 and Nanog, while overexpressing the astrocytic differentiation marker GFAP. On the other hand, DBTRG cells displayed a stem cell marker expression phenotype more pronounced as compared to U87 cells, featuring higher levels of all GSCs markers, as well as the NSCs markers oct-4 and Nanog. These phenotypical differences might be greatly influenced by the total number of time each cell line was divided for subculture, which was higher for the U87 cell line.

Finally, the expression of CD133 at the protein level was also assessed by flow cytometry using a specific anti-CD133 primary antibody (data not shown), however none of the analyzed subpopulations presented CD133 labeling, which we attributed to the fact that despite targeting different epitopes of the protein, the magnetic beads used for cell sorting might have caused a conformational change in CD133 structure or a stearic block that prevented the binding of the antibody.

Taken together, our results allowed us to conclude that the recurrent cell line DBTRG-05MG displays a more pronounced profile of malignancy, featuring a higher percentage of $CD133^+$ cells that exhibit a phenotype more close to a normal stem cell than the fraction isolated from the primary U87 cell line. Furthermore, we can also conclude that a cell cannot be defined as a GSC based only on the expression pattern of a single stem cell marker, particularly CD133, thus being essential to assess the expression profile of a group of stem cell markers in order to properly define a stemness phenotype.

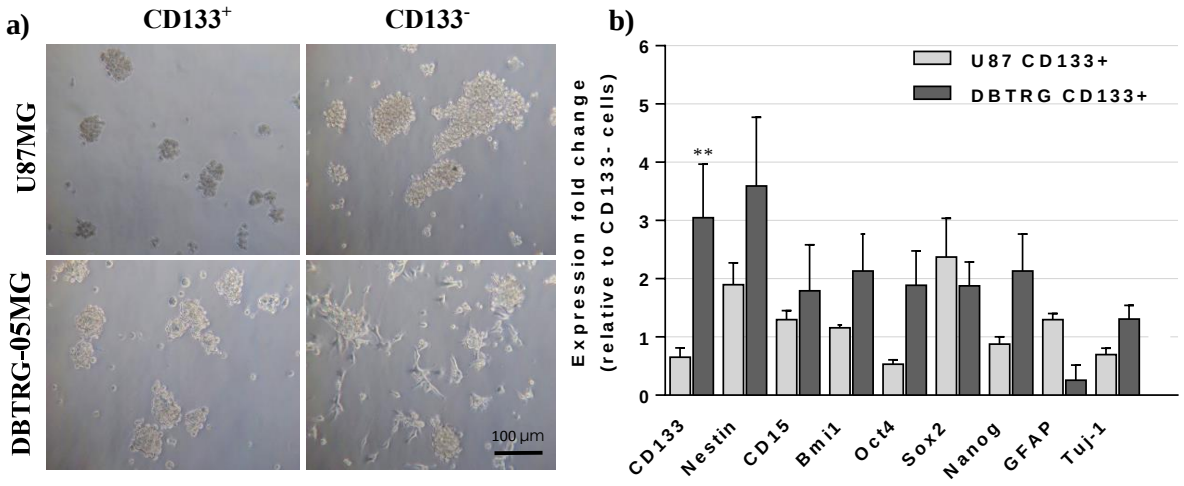

Figure 8 – Validation of CD133$^+$ cells as GSCs through the evaluation of their ability to form neurospheres and their stemness gene expression profiles. (a) - CD133+ and CD133- cells isolated from both U87MG and DBTRG-05MG cell lines were cultured for 4 days in serum-free DMEM-F12 supplemented with 20µg/mL of B27 and 0.02µg/mL of bFGF/EGF, in low-adherence plates in order to assess their ability to form floating neurospheres. **(b) –** Relative quantification of GSCs specific markers (CD133, Nestin, CD15, Bmi-1), NSCs markers (Oct-4, Sox2, Nanog) and astrocytic and neuronal lineage differentiation markers (GFAP and Tuj-1, respectively) in the CD133$^+$ and CD133$^-$ sorted fractions of both U87MG and DBTRG-05MG cell lines was performed by qRT-PCR. Results are expressed as fold change with respect to CD133$^-$ cells. Values correspond to the mean ± SD of 2-7 experiments. ** $p < 0.01$, compared to CD133$^-$ cells.

4.2 Evaluation of miR-29b expression in $CD133^+$/$CD133^-$ subpopulations isolated from a primary and a recurrent cell line

MicroRNAs are emerging as regulators of GBM initiation and progression, playing key roles in a wide range of biological processes that include cell proliferation, invasion and migration, as well as resistance to cellular apoptosis.[86] Recent studies have also highlighted the importance of these small RNA molecules in the maintenance of GSCs properties, mainly by controlling self-renewal, differentiation and cell fate.[87] However, so far, few studies have been conducted in order to elucidate how altered miRNA expression influences GSCs malignancy.

MiRNA-29b is a tumor suppressive microRNA that is frequently downregulated in GBM and hence often associated with a poor prognosis.[88] This miRNA is known to target the 3'UTR of proteins responsible for the regulation of epigenetic mechanisms, including the *de novo* DNA methyltransferases 3A and 3B and the transcription factor YY1, and signaling pathways that ultimately enhance cell migration and invasion, as is the case of PGRN.[89–91]

Therefore, in order to clarify the role of miR-29b in GSCs, we first examined its expression levels in the CD133-positive and negative subpopulations isolated from both U87 and DBTRG cell lines, using qRT-PCR.

As shown in figure 9 the expression pattern of this miRNA surprisingly remained constant between both $CD133^+$ and $CD133^-$ fractions isolated from the primary U87 cell line, which may be explained by the results obtained in the validation of U87 $CD133^+$ cells as GSCs, since they exhibited a profile more similar to a differentiated cell than that of a stem-like cell. On the other hand, miR-29b expression in DBTRG $CD133^+$ cells was significantly downregulated as compared to both their negative counterparts and U87 $CD133^+$ cells, which can also be correlated with the higher profile of malignancy displayed by these cells, in consequence of a more pronounced stem cell phenotype.

To further correlate the expression of miR-29b with that of its targets, we next assessed by qRT-PCR the expression level of PGRN, DNMT3A, DNMT3B and YY1 in the positive and negative fractions isolated from both cell lines. According to the obtained results, miR-29b expression pattern highly correlates with the expression of its targets: the expression levels of PGRN and YY1 in the CD133 positive fraction isolated from U87 cells were lower as compared to the negative fraction, although this decrease was not statistically significant, while both DNA methyltransferases 3A and 3B expression profile was similar to the one displayed by their negative counterparts, which was consistent with the similar expression pattern of miR-29b in

both subpopulations isolated from these cells. In the DBTRG cell line, all genes were overexpressed in the $CD133^+$ fraction, in particular DNMT3A and PGRN, with a fourfold and more than a twofold change as compared to the negative fraction, respectively. Since miR-29b is predicted to regulate the expression of these proteins by binding to the 3'UTR of their mRNA sequences, it should be expected that a decrease in miR-29b expression would be accompanied by an upregulation of its targets expression, as demonstrated by our results.

Finally, we also compared the expression of miR-29b in both CD133- positive and negative fractions isolated from the two GBM cell lines in culture, to primary human astrocytes (Figure 11). As expected, miR-29b was shown to be downregulated in both U87 and DBTRG CD133-sorted subpopulations, particularly in DBTRG $CD133^+$ cells, with respect to human astrocytes. This result can be easily correlated with the higher aggressiveness displayed by the DBTRG cell line.

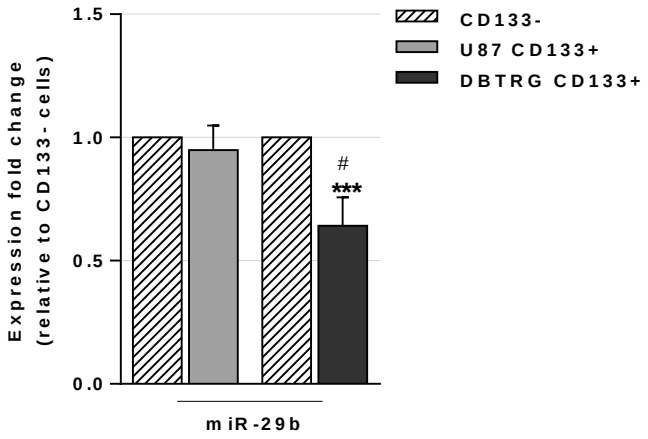

Figure 9 – miR-29b expression profile in CD133- positive and negative subpopulations isolated from a primary and a recurrent cell line, U87MG and DBTRG-05MG, respectively. The expression levels of miR-29b were measured by qRT-PCR in CD133- positive and negative cells isolated from both U87MG and DBTRG-05MG cell lines. The Pfaffl method was used to calculate the fold changes of miR-29b, with respect to the $CD133^-$ population of each cell line. Values correspond to the mean ± SD of 4-5 independent experiments. # $p < 0.05$, compared to U87 $CD133^+$ cells, *** $p < 0.001$, compared to $CD133^-$ cells.

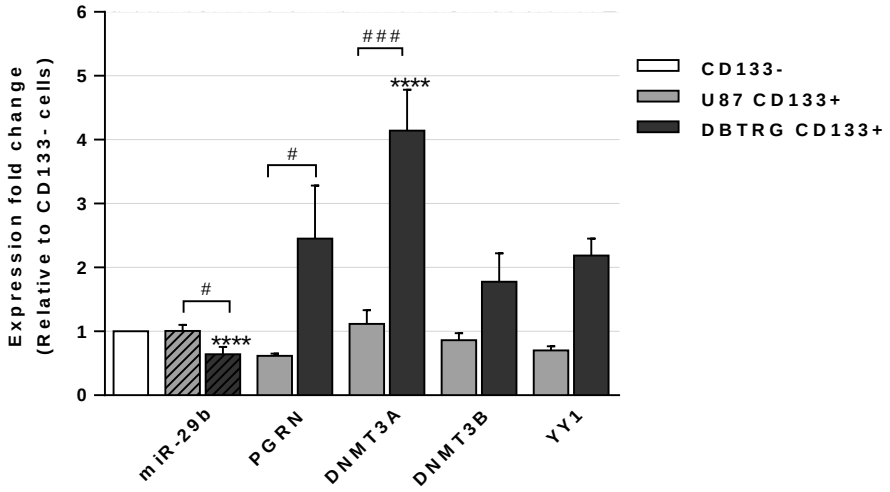

Figure 10 – miR-29b regulates the expression of several proteins involved in GBM pathogenesis. MiR-29b levels and PGRN, DNMT3A, DNMT3B and YY1 mRNA levels in CD133 subpopulations isolated from both U87MG and DBTRG-05MG cell lines were determined and correlated by qRT-PCR. Following qRT-PCR-mediated mRNA quantification, the Pfaffl method was used to calculate the mRNA fold changes of miR-29b and its respective targets, with respect to $CD133^-$ cells of each cell line. Values correspond to the mean ± SD of 5-7 independent experiments. # $p < 0.05$, ### $p < 0.001$, compared to U87 $CD133^+$ cells, ** $p < 0.01$, **** $p < 0.0001$, compared to $CD133^-$ cells.

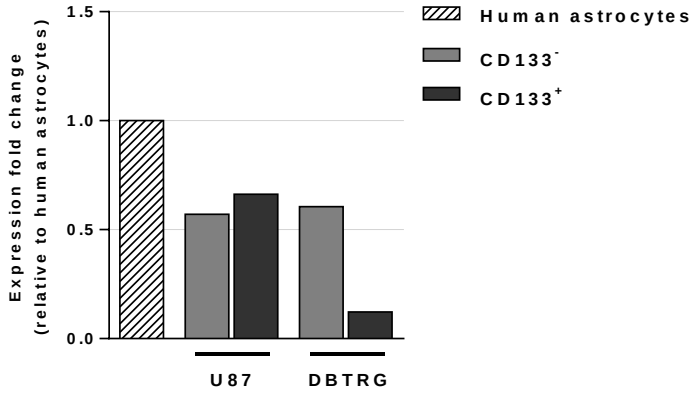

Figure 11 – miR-29b is downregulated in CD133- positive and negative subpopulations isolated from U87MG and DBTRG-05MG human GBM cell lines. The expression levels of miR-29b were measured by qRT-PCR in human astrocytes and in both CD133 positive and negative cell fractions isolated from U87 and DBTRG cells. The Pfaffl method was used to calculate the fold change of miR-29b with respect to human astrocytes. The results are representative of one experiment performed in duplicates.

4.3 Modulation of miR-29b expression in two different GBM cell lines through the delivery of a synthetic miR-29b mimic

Since miR-29b is downregulated in GBM and this expression pattern is in reverse correlation with the expression of proteins involved in key processes that drive GBM progression and aggressiveness, we next decided to evaluate the biological effect of restoring miR-29b expression, *per se*, or in combination with three chemotherapeutic agents, on cell viability. For this purpose we transfected both U87MG and DBTRG-05MG cells with a synthetic miR-29b mimic.

In order to optimize the delivery of miR-29b mimics to the GBM cell lines, we first assessed the transfection efficiency of three different delivery vectors: 1) a liposomal system composed of a mixture of DOGS and DOPE (DLS), 2) the commercial reagent DharmaFECT, and 3) a cell penetrating peptide, which is currently been evaluated in our laboratory as a transfection reagent for GBM gene therapy.

The choice of the delivery vehicle was based on its toxicity profile and concomitant reduction of cell viability, assessed by the AlamarBlue assay, as well as the post-transfection effects on the expression levels of both miR-29b and miR-29b targets, evaluated by qRT-PCT.

According to the obtained results (Figure 12), U87 cell transfection with the delivery liposomal system (DLS) resulted in a higher increase of miR-29b expression levels, with a fifty fold change with respect to control cells, instead of the twenty fold or five fold increase, produced by DharmaFECT or the cell penetrating peptide, respectively. Furthermore, transfection with DLS led to a significant decrease in DNMT3A expression, the gene with the higher expression in DBTRG $CD133^+$ cells (Figure 13). Nevertheless, we did not observe significant alterations in the mRNA levels of the other miR-29b target genes, neither using DLS nor DharmaFECT as transfection reagents. Unexpectedly, the association of the cell penetrating peptide with miR-29b mimics caused an increase in the expression of all genes. Although we did not observe a significant reduction in cell viability following transfection with DLS (data not shown), taken together, our results demonstrate that this system is the most efficient in delivering miR-29b mimics to cells in culture and completely correlates with previous observations made in our laboratory regarding other cell lines and miRNA mimics.

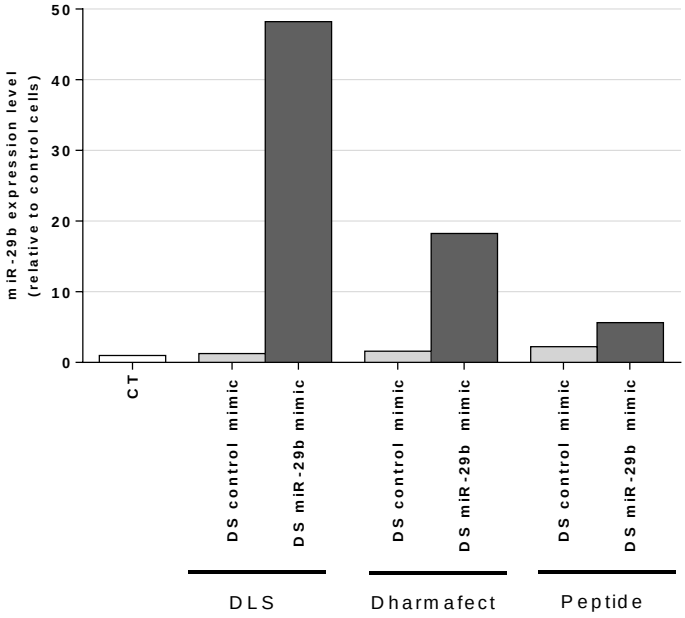

Figure 12 – Evaluation of miR-29b expression levels in U87 cells following transfection with miR-29b mimics. U87 cells were plated into 48-well plates at a cell density of $2x10^4$ cells/well. Twenty-four hours after plating cells were transfected for 4h, using three different delivery vehicles: DLS, DharmaFECT and a cell penetrating peptide under study in our laboratory. The transfection reagents were employed to deliver miR-29b mimics or a negative control oligonucleotide (control mimic), at a final concentration of 50nM. MiR-29b expression was assessed by qRT-PCR and the Pfaffl method was used to calculate fold changes of miR-29b expression, with respect to control untreated cells. Results are presented as miRNA fold change with respect to control untreated cells. The results are representative of one experiment performed in duplicates.

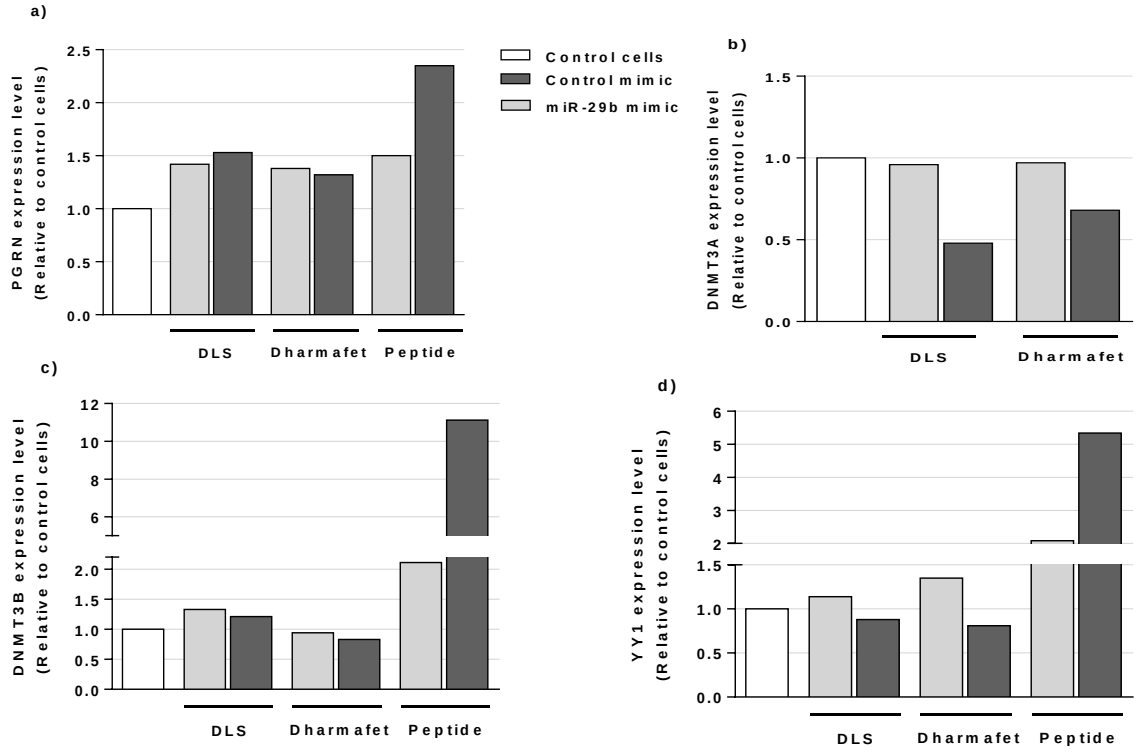

Figure 13 – Assessment of the expression levels of miR-29b targets in U87 cells, following transfection with miR-29b mimics. U87 cells were plated into 48-well plates at a cell density of $2x10^4$ cells/well. Twenty-four hours after plating cells were transfected for 4h with three different transfection reagents: DLS, DharmaFECT and a cell penetrating peptide currently under study in our laboratory. The transfection reagents were employed to deliver miR-29b mimics or a negative control oligonucleotide (control mimic), at a final concentration of 50nM. The expression levels of **(a)** PGRN, **(b)** DNMT3A, **(c)** DNMT3B and **(d)** YY1 were measured by qRT-PCR, following transfection and the Pfaffl method was used to calculate fold changes of gene expression for each gene, with respect to control untreated cells. The results are representative of one experiment performed in duplicates.

4.3.1. Reestablishment of miR-29b expression sensitizes GBM cells to tyrosine kinase inhibitors (sunitinib and axitinib) and TMZ

In order to evaluate whether a reestablishment of miR-29b expression levels would sensitize both U87MG and DBTRG-05MG cells to drug treatment, cell viability was measured after transfection with miR-29b mimics, either *per se*, or in combination with the tyrosine kinase inhibitors sunitinib and axitinib, or with temozolomide, a first-line drug for GBM treatment.

Both sunitinib and axitinib are selective inhibitors of tyrosine kinase receptors, including the VEGF and PDFG receptors, essential for sustaining angiogenesis during tumor progression and, therefore, considered a promising chemotherapeutic approach for highly vascularized tumors, such as GBM. These antiangiogenic drugs are currently approved by the FDA for treatment of gastro-intestinal stromal tumors as well as renal cell carcinoma, and are under phase II clinical trials for GBM treatment.[81,92]

Twenty-four hours after transfection with DLS- conjugated oligonucleotides (control oligonucleotides or miR-29b mimics), cells were incubated with each of the aforementioned drugs for 48 h, before the assessment of cell viability (Figure 14). Initially, we tested different concentrations of each of the drugs under study, in order to determine the optimal concentration of drug that displayed less unspecific effects and a better therapeutic response.

A significant decrease in cell viability was observed in U87 cells transfected with miR-29b mimics and further exposed to axitinib at a final concentration of 10μM and 20μM (the two concentrations that produced the best results), when compared to untreated control cells, or to control cells transfected with miR-29b mimics in the absence of any drug. Incubation with 600μM of TMZ after cell transfection with miR-29b oligonucleotides also reduced cell viability as compared to untreated control cells. Nevertheless, these results were found to be very similar following transfection with miR-29b mimics or control oligonucleotide, indicating a high degree of unspecificity. The observed effects are probably directly related with drug-induced toxicity or with an unspecific effect connected with the transfection process.

Similar to U87 cells, a significant reduction in cell viability was observed in DBTRG cells treated with axitinib at a concentration of 10μM, in comparison to control cells. Furthermore, a significant decrease was also reported in cells transfected with miR-29b mimics and treated with the highest concentration of axitinib, as compared to control cells or to control cells transfected with miR-29b oligonucleotides, as well as to cells transfected with miR-29b and further incubated with sunitinib at a concentration of 10μM. Nevertheless, we believe that

this effect is exclusively due to drug treatment and not a combination of both miR-29b transfection and drug exposure.

In general, and as demonstrated in figure 14, we did not observe a significant and specific reduction in cell viability neither after transfection with miR-29b mimics *per se* or in combination with the three tested chemotherapeutic drugs. This might be explained by an inefficient delivery of miR-29b mimics to the cultured cells, their entrapment in the endosomes, which could result in a poor silencing activity in the cytoplasm, or unexpected target competition, which could lead to preferential mimic binding to protein targets without therapeutic potential but with higher 3'UTR affinity towards miR-29b. Hence, we decided to test a different delivery vehicle, DharmaFECT, to clarify some of these hypothesis (Figure 15).

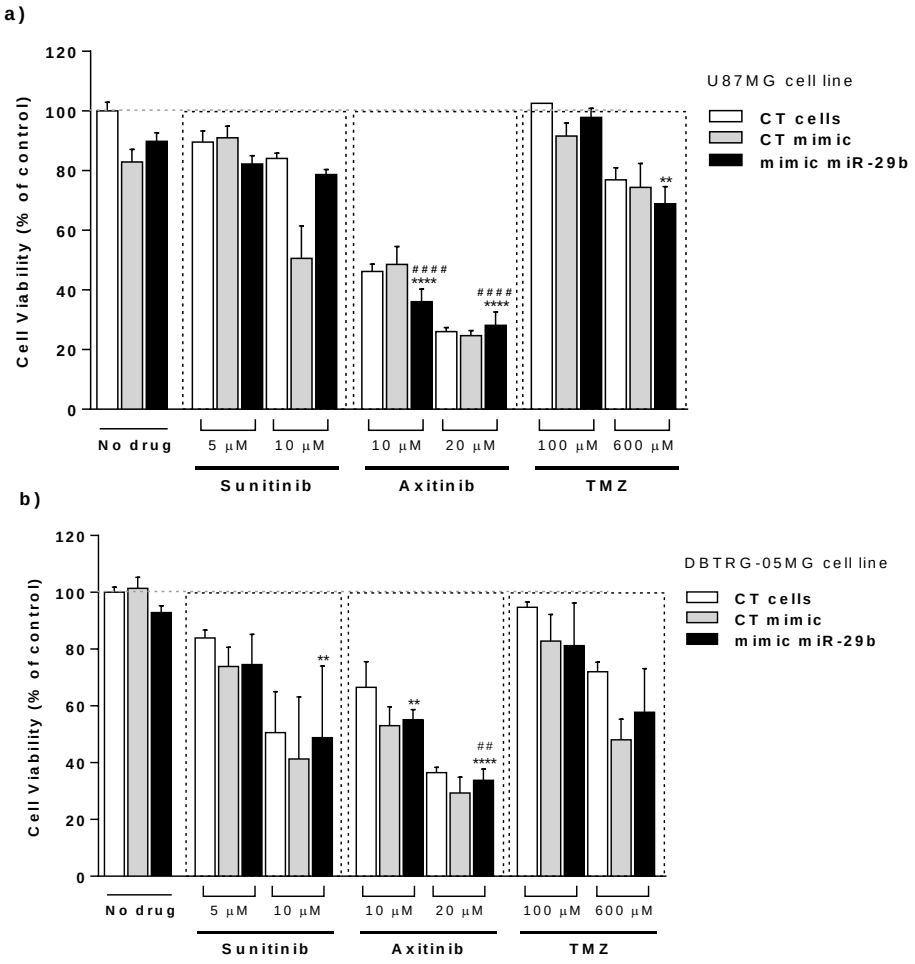

Figure 14 – Cell viability after incubation with sunitinib, axitinib or TMZ, either *per se* or in combination with miR-29b overexpression. Both **(a)** U87 and **(b)** DBTRG cells were plated into 48-well plates at a cell density of $2x10^4$ cells/well. Twenty-four hours after plating cells were transfected for 4h with DLS- conjugated either with a negative control oligonucleotide or miR-29b mimic (at a final concentration of 50nM per well), and further incubated with fresh medium for another 24h before drug exposure. Cells were subsequently incubated with sunitinib (5µM and 10µM), axitinib (10µM and 20µM) and TMZ (100µM and 600µM) for 48h, before assessment of cell by the AlamarBlue assay. ** $p < 0.01$, **** $p < 0.0001$, with respect to untreated control cells, ### $p < 0.001$, #### $p < 0.0001$ with respect to cells transfected with miR-29b oligonucleotides *per se*. Values are presented as mean ± SD and are representative of 3 independent experiments.

As shown in Figure 10, cell viability was significantly decreased when U87 cells were transfected with miR-29b oligonucleotides and further incubated with both sunitinib and axitinib at a final concentration of 5µM, in comparison with untreated control cells or control

cells exposed to each drug, respectively. However, miR-29b overexpression failed to sensitize U87 cells towards TMZ. Despite this fact, the reestablishment of miR-29b levels *per se*, decreased cell viability by approximately 30%, thus demonstrating that modulation of this particular miRNA, by itself, might also constitute a promising therapeutic approach towards GBM treatment.

The results obtained for the DBTRG cell line revealed that these cells appear to be more resistant to the cytotoxic effects of each of the three chemotherapeutic drugs, particularly TMZ, which is consistent with the literature and closely related to the fact that DBTRG is a recurrent cell line and hence displays a more aggressive phenotype, as well as intrinsic chemoresistance, as compared to a primary cell line, such as U87.

In addition, our results also demonstrated that transfection of DBTRG cells with miR-29b *per se*, reduces cell viability by approximately 40%, relative to control cells or cells transfected with the negative control oligonucleotide, and this effect is far more pronounced in these cells as compared to U87. This may be directly related to the fact that DBTRG also exhibit a more pronounced downregulation of miR-29b expression levels with respect to primary astrocytes.

Furthermore, miR-29b modulation in combination with sunitinib and axitinib reduced cell viability by approximately 30%, although this effect might be entirely attributed to miR-29b delivery and not to a synergistic effect resultant from this combination. Nevertheless, similarly to U87 cells, reestablishment of miR-29b expression failed to sensitize DBTRG cells to the effects of TMZ.

For most experimental conditions the negative control oligonucleotide failed to elicit an effect similar to the one observed with the miR-29b mimic, suggesting that the use of Dharmafect eliminated, for the most part, the unspecific effects previously observed with the DLS system. Overall, these results demonstrate a therapeutically relevant effect of miR-29b overexpression that can, in some cases, improve the cytotoxic effect induced by drug treatment, leading to significant reduction of cell viability.

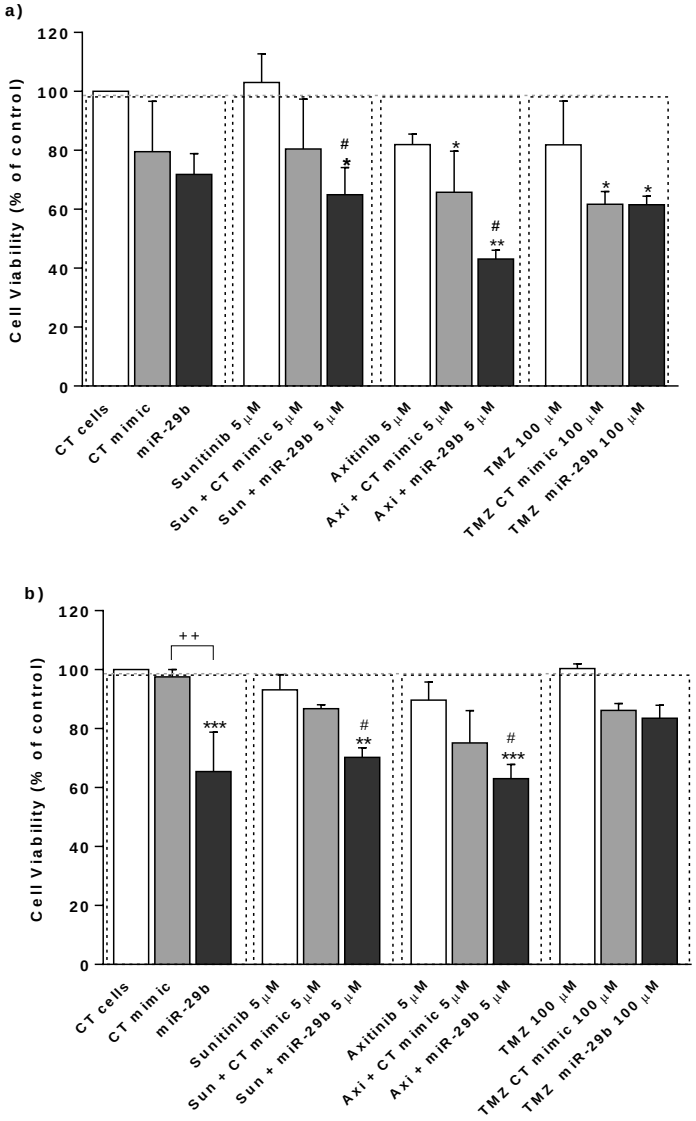

Figure 15 – Cell viability after incubation with sunitinib, axitinib or TMZ, either *per se* or in combination with transfection of miR-29b and scrambled oligonucleotides. Both **(a)** U87 and **(b)** DBTRG cells were plated into 48-well plates at a cell density of $2x10^4$ cells/well. Twenty-four hours after plating cells were transfected for 4h with DharmaFECT- conjugated either with a negative control oligonucleotide or miR-29b mimic (at a final concentration of 50nM per well), and further incubated with fresh medium for another 24h before drug exposure. Cells were subsequently incubated with sunitinib (5μM), axitinib (5μM) and TMZ (100μM) for 48h, before assessment of cell viability by the AlamarBlue assay. * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$, with respect to untreated control cells, # $p < 0.01$ with respect to control cells exposed to each drug *per se*, ++ $p < 0.01$, with respect to cells transfected with the negative control oligonucleotide. Values are presented as mean ± SD are representative of 3 independent experiments.

4.4 Reestablishment of miR-29b expression levels reduces global DNA methylation

Aberrant DNA methylation is a frequent event in many human cancers and has been proposed as a hallmark of tumorigenesis.[93,94] In GBM, DNA hypomethylation, which frequently results in genome instability and proto-oncogene formation, walks hand in hand with hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes, and their subsequent silencing. Genes involved in a wide range of biological processes, such as cell cycle control, DNA repair, tumor invasion and apoptosis, are now known to be silenced or downregulated in GBM, which has been closely related with GBM progression and poor prognosis.[95,96,97]

In order to investigate the effect of miR-29b modulation in the global DNA methylation status of GBM cells, we employed a specific ELISA assay to assess the levels of 5-methylcytosine (5-mC) in the genomic DNA of cells transfected either with a negative control oligonucleotide or a miR-29b mimic, and compared these levels to those of untreated control cells.

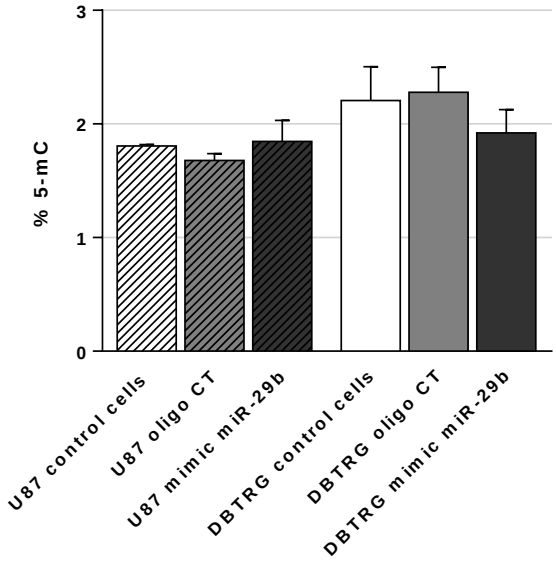

Figure 16 – Assessment of global DNA methylation levels of both U87MG and DBTRG-05MG cell lines following miR-29b modulation. The levels of 5-mC were quantified by ELISA using a specific anti-5-Methylcytosine monoclonal antibody. Results are expressed as the % of 5-mC and were determined through a logarithmic second-order regression of the standard curve. Values correspond to the mean ± SD of 3 independent experiments performed in duplicate.

According to the obtained results, DBTRG cells show a higher content of 5-mC when compared to U87 cells, which is consistent with the higher expression of the *de novo* DNA methyltransferases 3A and 3B observed in this study. Moreover, our results further indicate that a reestablishment of miR-29b expression cells in this cell line reduced, albeit not significantly, the percentage of 5-mC in DBTRG cells with respect to control cells and cells transfected with

the negative control oligonucleotide. No significant changes were observed in the methylation status of U87 cells following overexpression of miR-29b.

The observed changes in the methylation status of DBTRG may help explain the stronger therapeutic impact of miR-29b modulation observed in this cell line when compared to U87 cells.

CHAPTER 5 - Concluding remarks

5. Concluding remarks

In this work we presented a preliminary study on the role of miR-29b in GBM. Although much remains to be achieved, we are now more close to understanding the importance of this miRNA in the regulatory network that drives GBM initiation and progression. Moreover, our results point towards the feasibility of employing miRNA-modulation strategies, in combination with chemotherapy, as a new kind of therapeutic approach towards GBM treatment.

The main conclusions of our study are summarized below.

- The recurrent cell line DBTRG displays a stronger profile of malignancy, when compared to the primary U87 cell line. This profile is consistent with the higher percentage of $CD133^+$ GSCs observed in DBTRG cells, which promotes more pronounced stem cell-like properties in this cell line. Contrarily to DBTRG cells, U87 $CD133^+$ cells exhibit a phenotype more similar to that of a differentiated cell, which made us question not only the specificity of CD133 as a stem cell marker, but also the existence of GSCs in immortalized cell lines after extensive passaging;
- miR-29b is downregulated in both U87 and DBTRG cells, in comparison with human astrocytes. In addition, DBTRG $CD133^+$ cells express less miR-29b than their negative counterparts, which helps to explain the more aggressive phenotype displayed by the positive fraction. However, miR-29b expression in both CD133 positive and negative subpopulations isolated from U87 cells appears to be similar, which supports the absence of differences between the phenotypes of $CD133^+$ cells and $CD133^-$ cells isolated from this cell line;
- The expression of PGRN, DNMT3A, DNMT3B and YY1, all validated targets of miR-29b, inversely correlates with miR-29b expression profile in DBTRG $CD133^+$ cells. No differences can be observed in both the CD133 negative and positive fractions isolated from U87 cells, which also correlates with the similar expression of miR-29b present in these two subpopulations;
- Reestablishment of miR-29b expression levels sensitizes U87 cells to the cytotoxic effects of sunitinib and axitinib, while the DBTRG cell line displays a more resistant

phenotype to chemotherapy. However, this cell line presents a decrease in cell viability following overexpression of miR-29b *per se*.

- Modulation of miR-29b expression decreases the global DNA methylation status of DBTRG cells. However, no significant changes in the content of 5-mC are observed in U87 cells, following reintroduction of miR-29b, which correlates with the lower content of 5-mC and DNA methyltransferases observed in this cell line, with respect to DBTRG.

Taken together our results demonstrate that miR-29b might constitute a promising target for a multimodal therapeutic strategy based on miRNA modulation in combination with antiangiogenic chemotherapy in primary GBMs, while it can also mediate significant toxicity *per se* in recurrent tumors, resistant to standard treatment.

CHAPTER 6 - Bibliographic references

1. Mathers, C., Fat, D. & Boerma, J. The global burden of disease: 2004 update. (2008).
2. Jemal, A., Bray, F. & Center, M. Global cancer statistics. CA **61,** 69–90 (2011).
3. Lazebnik, Y. What are the hallmarks of cancer? Nat. Rev. Cancer **10,** 232–233 (2010).
4. Hanahan, D. & Weinberg, R. The hallmarks of cancer. Cell **100,** 57–70 (2000).
5. Hanahan, D. & Weinberg, R. a. Hallmarks of cancer: the next generation. Cell **144,** 646–74 (2011).
6. Schwartzbaum, J. a, Fisher, J. L., Aldape, K. D. & Wrensch, M. Epidemiology and molecular pathology of glioma. Nat. Clin. Pract. Neurol. **2,** 494–503; quiz 1 p following 516 (2006).
7. Omuro, A. & DeAngelis, L. M. Glioblastoma and other malignant gliomas: a clinical review. Jama **310,** 1842–50 (2013).
8. Louis, D. N. et al. The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol. **114,** 97–109 (2007).
9. States, C. B. T. R. of the U. CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2004-2006. (2010).
10. Wesseling, P. Emerging Concepts in Neuro-Oncology. 3–20 (2013). doi:10.1007/978-0-85729-458-6
11. Thakkar, J. P. et al. Epidemiologic and molecular prognostic review of glioblastoma. Cancer Epidemiol. Biomarkers Prev. **23,** 1985–96 (2014).
12. Luo, J., Wang, X., Yang, Y. & Mao, Q. Role of micro-RNA (miRNA) in pathogenesis of glioblastoma. Eur. Rev. 1630–1639 (2015).
13. Preusser, M. et al. Current concepts and management of glioblastoma. Ann. Neurol. **70,** 9–21 (2011).
14. Alifieris, C. & Trafalis, D. T. Glioblastoma Multiforme: Pathogenesis and Treatment. Pharmacol. Ther. (2015).
15. Adamson, C., Kanu, O., Mehta, A., Di, C. & Lin, N. Glioblastoma multiforme: a review of where we have been and where we are going. Expert Opin. Investig. Drugs 1061–1084 (2009).
16. Fisher, J. L., Schwartzbaum, J. a, Wrensch, M. & Wiemels, J. L. Epidemiology of brain tumors. Neurol. Clin. **25,** 867–90, vii (2007).
17. Kleihues, P. & Ohgaki, H. Primary and secondary glioblastomas: from concept to clinical diagnosis. Neuro. Oncol. 44–51 (1999).
18. Ohgaki, H. & Kleihues, P. The definition of primary and secondary glioblastoma. Clin. Cancer Res. **19,** 764–72 (2013).
19. Ohgaki, H. & Kleihues, P. Genetic pathways to primary and secondary glioblastoma. Am. J. Pathol. **170,** 1445–53 (2007).
20. Anton, K., Baehring, J. M. & Mayer, T. Glioblastoma multiforme: overview of current treatment and future perspectives. Hematol. Oncol. Clin. North Am. **26,** 825–53 (2012).
21. Khasraw, M. & Lassman, A. B. Advances in the treatment of malignant gliomas. Curr. Oncol. Rep. **12,** 26–33 (2010).

22. Stupp, R. & Mason, W. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. Engl. J. 987–996 (2005).

23. Friedman, H., Kerby, T. & Calvert, H. Temozolomide and treatment of malignant glioma. Clin. Cancer Res. **6,** 2585–2597 (2000).

24. Zhang, J. Temozolomide: mechanisms of action, repair and resistance. Curr. Mol. 102–114 (2012).

25. Cabrini, G., Fabbri, E., Lo Nigro, C., Dechecchi, M. & Gambari, R. Regulation of expression of O6-methylguanine-DNA methyltransferase and the treatment of glioblastoma (Review). Int. J. Oncol. 1–12 (2015).

26. Tirino, V. et al. Cancer stem cells in solid tumors: an overview and new approaches for their isolation and characterization. FASEB J. **27,** 13–24 (2012).

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

28. Visvader, J. E. & Lindeman, G. J. Cancer stem cells: current status and evolving complexities. Cell Stem Cell **10,** 717–28 (2012).

29. Glorioso, J. C. & Fink, D. J. Looking ahead in cancer stem cell research. Mol. Ther. **17,** 13–18 (2008).

30. Dick, D. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat. Med (1997).

31. Al-Hajj, M., Becker, M. W., Wicha, M., Weissman, I. & Clarke, M. F. Therapeutic implications of cancer stem cells. Curr. Opin. Genet. Dev. **14,** 43–7 (2004).

32. Bjerkvig, R., Tysnes, B. & Aboody, K. The origin of the cancer stem cell: current controversies and new insights. Nat. Rev. **5,** 899–904 (2005).

33. Jordan, C., Guzman, M. & Noble, M. Cancer stem cells. New Engl. J. … 1253–1261 (2006).

34. Singh, S., Hawkins, C., Clarke, I. & Squire, J. Identification of human brain tumour initiating cells. Nature **432,** (2004).

35. Tabatabai, G. & Weller, M. Glioblastoma stem cells. Cell Tissue Res. **343,** 459–65 (2011).

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

37. Fael Al-Mayhani, T. M. et al. An efficient method for derivation and propagation of glioblastoma cell lines that conserves the molecular profile of their original tumours. J. Neurosci. Methods **176,** 192–9 (2009).

38. 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).

39. Gilbertson, R. & Rich, J. Making a tumour's bed: glioblastoma stem cells and the vascular niche. Nat. Rev. Cancer **7,** 733–736 (2007).

40. Liu, G. et al. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. Mol. Cancer **5,** 67 (2006).

41. Li, Z., Wang, H., Eyler, C. E., Hjelmeland, A. B. & Rich, J. N. Turning cancer stem cells inside out: an exploration of glioma stem cell signaling pathways. J. Biol. Chem. **284,** 16705–9 (2009).

42. Kalkan, R. Therapeutic Potential of Glioblastoma Stem Cells. MOJ Cell Sci Rep **2,** 24–29 (2015).

43. Binda, E., Reynolds, B. a. & Vescovi, a. L. Glioma stem cells: turpis omen in nomen? (the evil in the name?). J. Intern. Med. **276,** 25–40 (2014).

44. Brower, J. V, Clark, P. a, Lyon, W. & Kuo, J. S. MicroRNAs in cancer: glioblastoma and glioblastoma cancer stem cells. Neurochem. Int. **77,** 68–77 (2014).

45. Yang, H. W., Xing, H. & Johnson, M. D. A major role for microRNAs in glioblastoma cancer stem-like cells. Arch. Pharm. Res. **38,** 423–34 (2015).

46. Bandey, I., Chiou, S.-H., Huang, a-P., Tsai, J.-C. & Tu, P. Progranulin promotes Temozolomide resistance of glioblastoma by orchestrating DNA repair and tumor stemness. Oncogene **34,** 1853–64 (2015).

47. Wongtrakoongate, P. Epigenetic therapy of cancer stem and progenitor cells by targeting DNA methylation machineries. World J. Stem Cells **7,** 137–48 (2015).

48. Garzon, R., Calin, G. a. & Croce, C. M. MicroRNAs in Cancer. Annu. Rev. Med. **60,** 167–179 (2009).

49. Plaisier, C., Pan, M. & Baliga, N. A miRNA-regulatory network explains how dysregulated miRNAs perturb oncogenic processes across diverse cancers. Genome Res. 2302–2314 (2012).

50. Volinia, S. et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc. Natl. Acad. Sci. U. S. A. **103,** 2257–61 (2006).

51. Dong, H. et al. MicroRNA: function, detection, and bioanalysis. Chem. Rev. (2013).

52. Sato, F., Tsuchiya, S., Meltzer, S. J. & Shimizu, K. MicroRNAs and epigenetics. FEBS J. **278,** 1598–609 (2011).

53. Ha, M. & Kim, V. N. Regulation of microRNA biogenesis. Nat. Rev. Mol. Cell Biol. **15,** 509–524 (2014).

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

55. Lin, S. & Gregory, R. I. MicroRNA biogenesis pathways in cancer. Nat. Rev. Cancer **15,** 321–333 (2015).

56. Várallyay, E., Burgyán, J. & Havelda, Z. MicroRNA detection by northern blotting using locked nucleic acid probes. Nat. Protoc. **3,** 190–6 (2008).

57. Calin, G. & Dumitru, C. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc. Natl. Acad. Sci. **99,** 13–18 (2002).

58. Mendell, J. T. & Olson, E. N. MicroRNAs in stress signaling and human disease. Cell **148,** 1172–87 (2012).

59. Di Leva, G. & Croce, C. M. Roles of small RNAs in tumor formation. Trends Mol. Med. **16,** 257–67 (2010).

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

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

62. Hong, Q., Fang, J., Pang, Y. & Zheng, J. Prognostic value of the microRNA-29 family in patients with primary osteosarcomas. Med. Oncol. **31,** 37 (2014).

63. Liston, A., Papadopoulou, A. S., Danso-Abeam, D. & Dooley, J. MicroRNA-29 in the adaptive immune system: setting the threshold. Cell. Mol. Life Sci. **69,** 3533–41 (2012).

64. Noguchi, M., Ropars, V., Roumestand, C. & Suizu, F. Proto-oncogene TCL1: more than just a coactivator for Akt. FASEB J. 2273–2284 (2007).

65. Jiao, J., Herl, L. D., Farese, R. V & Gao, F.-B. MicroRNA-29b regulates the expression level of human progranulin, a secreted glycoprotein implicated in frontotemporal dementia. PLoS One **5,** e10551 (2010).

66. Amodio, N., Leotta, M. & Bellizzi, D. DNA-demethylating and anti-tumor activity of synthetic miR-29b mimics in multiple myeloma. Oncotarget (2012).

67. Ahmed, Z. & Mackenzie, I. Progranulin in frontotemporal lobar degeneration and neuroinflammation. J. Neuroinflammation **13,** 1–13 (2007).

68. Toh, H., Chitramuthu, B. P., Bennett, H. P. J. & Bateman, A. Structure, function, and mechanism of progranulin; the brain and beyond. J. Mol. Neurosci. **45,** 538–48 (2011).

69. Ong, C. & Bateman, A. Progranulin (Granulin-epithelin precursor, PC-cell derived growth factor, Acrogranin) in proliferation and tumorigenesis. Histol. Histopathol. 1275–1288 (2003).

70. Wang, M., Li, G., Yin, J., Lin, T. & Zhang, J. Progranulin overexpression predicts overall survival in patients with glioblastoma. Med. Oncol. **29,** 2423–31 (2012).

71. He, Z., Ismail, A. & Kriazhev, L. Progranulin (PC-cell-derived growth factor/acrogranin) regulates invasion and cell survival. Cancer Res. 5590–5596 (2002).

72. Kanwal, R. & Gupta, S. Epigenetic modifications in cancer. Clin. Genet. **81,** 303–311 (2012).

73. Nagarajan, R. P. & Costello, J. F. Epigenetic mechanisms in glioblastoma multiforme. Semin. Cancer Biol. **19,** 188–97 (2009).

74. Dawson, M. a & Kouzarides, T. Cancer epigenetics: from mechanism to therapy. Cell **150,** 12–27 (2012).

75. Hervouet, E., Vallette, F. M. & Cartron, P.-F. Impact of the DNA methyltransferases expression on the methylation status of apoptosis-associated genes in glioblastoma multiforme. Cell Death Dis. **1,** e8 (2010).

76. Martinez, R., Martin-Subero, J. & Rohde, V. A microarray-based DNA methylation study of glioblastoma multiforme. Epigenetics **2,** 255–264 (2009).

77. Rajendran, G. et al. Epigenetic regulation of DNA methyltransferases: DNMT1 and DNMT3B in gliomas. J. Neurooncol. **104,** 483–94 (2011).

78. Tivnan, A. & McDonald, K. L. Current progress for the use of miRNAs in glioblastoma treatment. Mol. Neurobiol. **48,** 757–68 (2013).

79. Zhang, Y., Wang, Z. & Gemeinhart, R. a. Progress in microRNA delivery. J. Control. Release **172,** 962–74 (2013).

80. 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).

81. 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).

82. Trabulo, S., Resina, S., Simões, S., Lebleu, B. & Pedroso de Lima, M. C. A non-covalent strategy combining cationic lipids and CPPs to enhance the delivery of splice correcting oligonucleotides. J. Control. Release **145,** 149–58 (2010).

83. Wicha, M. S., Liu, S. & Dontu, G. Cancer stem cells: an old idea--a paradigm shift. Cancer Res. **66,** 1883–90; discussion 1895–6 (2006).

84. Sampetrean, O. & Saya, H. Characteristics of glioma stem cells. Brain Tumor Pathol. **30,** 209–14 (2013).

85. Fatoo, A. et al. Understanding the role of tumor stem cells in glioblastoma multiforme: a review article. J. Neurooncol. **103,** 397–408 (2011).

86. Costa, P. M. & Pedroso de Lima, M. C. MicroRNAs as Molecular Targets for Cancer Therapy: On the Modulation of MicroRNA Expression. Pharmaceuticals (Basel). **6,** 1195–220 (2013).

87. Liu, S. et al. Regulatory roles of miRNA in the human neural stem cell transformation to glioma stem cells. J. Cell. Biochem. **115,** 1368–80 (2014).

88. Amodio, N. et al. miR-29s : a family of epi-miRNAs with therapeutic implications in hematologic malignancies EPI-MIRNAS. (2015).

89. Garzon, R. et al. MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. **113,** 6411–6419 (2015).

90. Cortez, M. A. et al. miR-29b and miR-125a Regulate Podoplanin and Suppress Invasion in Glioblastoma. **990,** 981–990 (2010).

91. Jiao, J., Herl, L. D., Farese, R. V & Gao, F.-B. MicroRNA-29b regulates the expression level of human progranulin, a secreted glycoprotein implicated in frontotemporal dementia. PLoS One **5,** e10551 (2010).

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

93. Keshet, I. et al. Evidence for an instructive mechanism of de novo methylation in cancer cells. Nat. Genet. **38,** 149–53 (2006).

94. Esteller, M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene **21,** 5427–40 (2002).

95. Zhang, Z. et al. MiR-185 Targets the DNA Methyltransferases 1 and Regulates Global DNA Methylation in human glioma. 1–16 (2011).

96. Eramo, A. et al. Inhibition of DNA methylation sensitizes glioblastoma for tumor necrosis factor-related apoptosis-inducing ligand-mediated destruction. Cancer Res. **65,** 11469–77 (2005).

97. Mueller, W. et al. Downregulation of RUNX3 and TES by hypermethylation in glioblastoma. Oncogene **26,** 583–93 (2007).

98. Fan, C.-H. et al. O6-methylguanine DNA methyltransferase as a promising target for the treatment of temozolomide-resistant gliomas. Cell Death Dis. **4,** e876 (2013).