**Universidade do Minho**
Escola de Medicina

Joana Isabel Martins Cosme Vieira de Castro

# Interplay between molecular and cellular players on glioma pathophysiology

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# Interplay between molecular and cellular players on glioma pathophysiology

Tese de Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação do
**Doutor Bruno M. Costa**
e do
**Doutor António Salgado**

agosto de 2017

# DECLARAÇÃO

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# Acknowledgements / Agradecimentos

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# Abstract / Resumo

# Abstract

Malignant gliomas are the most common primary brain tumors, accounting for 80% of all neoplasms of the central nervous system, of which glioblastoma (GBM) is the most aggressive and deadly subtype. Even with a multimodal therapy approach that includes surgery and chemo-radio-therapy, the prognosis of glioma patients remains very poor. Moreover, the etiology and clinically-relevant prognostic factors in glioma remain largely undetermined. In this context, the research summarized in this thesis focuses on i) evaluating the influence of *TGF-β1* genetic variants in glioma susceptibility and patient prognosis; ii) identifying novel defining characteristics of glioma stem cells (GSCs), a subpopulation of cells that plays critical roles on tumor initiation, resistance and recurrence; and iii) unveiling new insights on the influence of mesenchymal stem cells (MSCs) in glioma behavior.

Many genetic polymorphisms have been associated with glioma susceptibility and prognosis. Several studies demonstrated that polymorphisms in the *TGF-β1* gene were associated with the susceptibility for different tumor types. Transforming growth factor beta (TGF-β) is known to play an important role in carcinogenesis, and its activity has been associated with poor prognosis in glioma patients. Taking into account that the relevance of single nucleotide polymorphisms (SNPs) in *TGF-β1* in glioma is not known, we evaluated two SNPs (-509C/T and 869T/C) in this gene in glioma risk and patient prognosis. A case-control study involving 138 Caucasian cancer-free control and 118 glioma patients from Portugal was performed. We showed that *TGF-β1* -509C/T and 869T/C variants were not significantly associated with glioma risk. Importantly, we demonstrated that both homozygous -509TT and 869CC genotypes were associated with longer overall survival of GBM patients. Our data suggested that *TGF-β*1 -509C/T and 869T/C polymorphisms may be relevant prognostic biomarkers in GBM patients.

A striking characteristic of malignant gliomas, particularly GBMs, is their highly heterogeneous and therapy-resistant nature. These features are partly attributed to GSCs, a subpopulation of cancer cells with stem cell features that are involved in tumor initiation, progression, and recurrence, making them crucial therapeutic targets. Their isolation has been challenging as the markers typically used lack sufficient specificity and sensitivity. Thus, we also investigated here if intracellular autofluorescence, a biomarker of epithelial cancer stem cells, could be used as a biomarker to improve GSCs identification and isolation. We found that both established and patient-derived primary GBM cells presented a subpopulation of autofluorescent cells (Fluo$^+$). Moreover, we showed that Fluo$^+$ cells had typical features of GSCs, including higher expression of stem cell protein markers and pluripotency-associated genes, enriched capacity to grow as neurospheres, and long-term self-renewal ability.

Additionally, treatments with temozolomide (TMZ) or radiation led to a significant increase in the percentage of Fluo$^+$ cells. Importantly, *in vivo* studies showed that mice with intracranial tumors derived from Fluo$^+$ GBM cells presented a significantly shorter overall survival than those with non-autofluorescent cells (Fluo$^-$) GBM cells. Finally, the underlying mechanism of the autofluorescent phenotype was due to the uptake, and accumulation of riboflavin in GSCs, by the ABCG2 transporters, exclusively in cells with GSCs' features. Together, our data revealed autofluorescence as a novel and useful biomarker for GSCs.

In the light of the lack of curative therapies for malignant glioma, many exploratory therapeutic strategies have been analyzed. Among these, MSCs have been studied as a new approach for the treatment of malignant gliomas, due to their inherent capacity of homing to glioma, and their ability to be engineered to deliver anti-tumoral agents. In the third experimental work included in this thesis, we assessed the impact of the secretome of MSCs on hallmark characteristics of GBM cells, such as cell viability, migration, proliferation, tumor growth, as well as chemotherapy response. Using *in vitro* approaches, we observed that GBM cells exposed to conditioned media (CM) from human umbilical cord perivascular cells (HUCPVCs, a MSC population) presented an increased cellular viability, proliferation and migration, while not affecting the sensitivity of GBM cells to TMZ treatment. Additionally, in the *in vivo* CAM assay, we found that CM from HUCPVCs promoted GBM tumor growth. Finally, proteomic analyses to characterize the secretome of HUCPVCs identified several proteins involved in promotion of cell survival, proliferation and migration, revealing novel putative molecular mediators for the effects observed in GBM cells exposed to HUCPVCs CM. Our data highlights that caution must be taken regarding the use of MSCs as stem-cell based therapies for GBM.

In summary, the thesis presented here contributes to the better understanding of several dimensions of glioma, from factors that may be causative, to those that influence their pathophysiology and progression, to therapeutics insights. Particularly, assessing *TGF-β1* SNPs -509C/T and 869T/C variants may be clinically relevant for GBM patients; using autofluorescence as a biomarker for GSCs identification may be important to develop new GSCs-specific therapies; and using MSCs as stem cell-based therapies for GBM does not seem to be a safe choice.

## Resumo

Os gliomas malignos são os tumores primários do cérebro mais frequentes, constituindo cerca de 80% de todas as neoplasias do sistema nervoso central, sendo o glioblastoma (GBM) o subtipo mais agressivo e letal. Mesmo com as abordagens terapêuticas usadas, que incluem cirurgia, radioterapia e quimioterapia, o prognóstico de doentes com glioma continua bastante crítico. Além disso, a etiologia e factores clínicos de prognóstico relevantes dos gliomas são praticamente desconhecidos. Neste contexto, os estudos sumarizados nesta tese pretenderam: i) avaliar a influência das variantes genéticas do gene *TGF-β1* na susceptibilidade para glioma e no prognóstico dos pacientes com glioma; ii) identificar novas características das células estaminais de glioma (*GSCs*), uma subpopulação de células com um papel importante na iniciação, resistência e recorrência dos tumores; e iii) revelar novos conhecimentos que a influência das células estaminais mesenquimatosas (*MSCs*) têm no comportamento dos GBMs.

Muitos polimorfismos genéticos têm sido associados com susceptibilidade para glioma e com prognóstico de pacientes com glioma. Vários estudos demonstraram que polimorfismos do gene *TGF-β1* estavam associados com a susceptibilidade para vários tipos tumorais. O *transforming growth factor beta* (TGF-β) tem um papel importante na carcinogénese e a sua atividade foi associada a um pior prognóstico de pacientes com glioma. Tendo em conta que a relevância de polimorfismos do tipo *single nucleotide polymorphism* (*SNPs*) do *TGF-β1* em gliomas não é conhecida, nós avaliamos a possível associação de dois *SNPs* neste gene (-509C/T e 869T/C) no risco e prognóstico de gliomas. Num estudo de caso-controlo demonstrámos que apesar de nenhum destes polimorfismos do *TGF-β1* estar associado com um maior risco de desenvolvimento de glioma, ambos os genótipos homozigóticos -509TT e 869CC estavam associados a uma maior sobrevida de pacientes com GBM. Os nossos resultados sugerem que os polimorfismos -509C/T e 869T/C do *TGF-β1* podem ser considerados biomarcadores de prognóstico em pacientes com GBM.

Uma característica impressionante dos gliomas, em particular dos GBMs, é a sua elevada heterogeneidade e resistência à terapia. Estas características são, em parte, atribuídas à presença de *GSCs*, que estão envolvidas na iniciação, progressão e recorrência tumoral, tornando-as um importante alvo terapêutico. O isolamento das *GSCs* tem constituído um desafio uma vez que os marcadores tipicamente usados não são específicos. Assim, nós também investigámos se a autofluorescência, um marcador usado na identificação de células estaminais cancerígenas de tumores epiteliais, poderia ser usado como novo biomarcador para melhor identificar e isolar as *GSCs*. Os nossos resultados demonstraram que linhas primárias e estabelecidas de GBM continham células autofluorescentes ($Fluo^+$). Mais ainda, verificámos que as células $Fluo^+$ apresentavam características típicas de *GSCs*, tais como uma elevada expressão de marcadores de células estaminais e de genes de pluripotencia, maior capacidade para crescer em neuro-esferas e uma capacidade de auto-renovação mais prolongada. Após tratamento com temozolomida (TMZ) e radiação verificou-se um aumento da percentagem de células Fluo$^+$. Para além disso, estudos *in vivos* revelaram que murganhos injetados intracranialmente com células de GBM Fluo$^+$ apresentavam uma sobrevida significativamente menor do que murganhos injetados com células de GBM Fluo$^-$. Por fim verificámos que o mecanismo subjacente ao fenótipo da autofluorescência era exclusivo das *GSCs* e se devia ao transporte de riboflavina, pelos transportadores ABCG2, e à sua acumulação intracelular. Todas estas evidências revelaram claramente que a autofluorescência é um novo biomarcador para a identificação de *GSCs*.

Tendo em conta a falta de terapias curativas no tratamento de glioma, muitas estratégias terapêuticas exploratórias têm sido analisadas. Entre estas, as *MSCs* têm sido estudadas como uma nova abordagem terapêutica no tratamento dos gliomas, uma vez que são capazes de migrar para os gliomas e podem ser facilmente modificadas para distribuir agentes anti-tumorais. No terceiro trabalho apresentado nesta tese, avaliámos o impacto do secretoma das *MSCs* em características de agressividade das células de GBM. Estudos *in vitro* demonstraram que células de GBM expostas a meios condicionados (*CM*) provenientes de células perivasculares humanas do cordão umbilical (*HUCPVCs*, uma população de *MSCs*) apresentavam um aumento na viabilidade, proliferação e migração celulares. Por outro lado, nenhum efeito foi observado na resposta à *TMZ*. Mais ainda, no ensaio *in vivo* da CAM, verificámos que o *CM* das *HUCPVCs* promovia o crescimento tumoral. Finalmente, a caracterização do secretoma das *HUCPVCs* foi efectuada por análises de proteómica, o que permitiu identificar varias proteínas envolvidas na sobrevivência, proliferação e migração das células de GBM, revelando novos e putativos mediadores moleculares envolvidos no efeito observado nas células de GBM quando expostas ao *CM* das *HUCPVCs*. Os nossos resultados demonstraram que é necessária alguma precaução no uso de *MSCs* como potenciais agentes terapêuticos para o tratamento de GBM.

Em suma, a tese aqui apresentada contribui para uma melhor compreensão de várias dimensões dos gliomas, desde fatores que possam ser causadores da doença, passando por fatores que influenciam a sua patofisiologia e progressão, até a perspetivas terapêuticas. Particularmente, avaliar os níveis dos SNPs -509C/T and 869T/C do *TGF-β1* pode ser clinicamente relevante para pacientes com GBM; usar a autofluorescência como biomarcador para identificar e isolar as *GSCs* poderá ser importante para desenvolver terapias específicas anti-*GSCs*; e usar *MSCs* como uma nova abordagem terapêutica no tratamento dos GBMs não aparenta ser uma escolha segura.

# Contents

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# List of Abbreviations

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**2-HG**: 2-Hydroxyglutarate
**5mc:** 5-methylcytosine
**ABC:** ATP-Binding Cassette
**ABCG2:** ATP-Binding Cassette sub-family G member 2
**ACN**: Acetonitrile
**ACTN4**: Alpha-actinin-4
**ADAM10**: Disintegrin and metalloproteinase domain-containing protein 10
**ALT:** Alternative Lengthening of Telomeres
**AMH:** Anti-Mullerian Hormone
**ARPC5**: Actin-Related Protein 2/3 Complex Subunit 5
**ASCs:** Adipose tissue-derived mesenchymal Stem Cells
**ATP:** Adenosine Triphosphate
**ATRX**: chromatin remodeler, formerly Alfa Thalassemia/mental Retardation syndrome X-linked
**BBB:** Blood–Brain Barrier
**BCRP:** Breast Cancer Resistance Protein
**b-FGF**: basic Fibroblast Growth Factor
**BM-MSCs:** Bone Marrow-derived Mesenchymal Stem Cells
**BMPs:** Bone Morphogenetic Proteins
**BMSCs:** Bone Marrow Stem Cells
**BTSCs**: Brain Tumor Stem Sells
**CCDC26:** Coiled-Coil Domain Containing 26
**CCL2**: C-C motif chemokine 2
**CCND1/CCNH -** Cyclin D1/ Cyclin H
**CD**: Cytosine Deaminase
**CDK4/CDK6**: Cyclin-Dependent Kinase 4/ Cyclin-Dependent Kinase 6
**CDKN2A/CDKN2B**: Cyclin-Dependent Kinase inhibitor 2A/ Cyclin-Dependent Kinase inhibitor 2B
**cDNA:** Complementary Deoxyribonucleic Acid
**CE:** Carboxylesterase
**CES**: Collision energy spread

**CI**: Confidence Intervals

**CIC:** homolog of the Drosophila gene capicua

**CM:** Conditioned Medium

**CNS**: Central Nervous System

**CpG**: Cytosine-phosphate-Guanine

**CSCs**: Cancer Stem Cells

**CTL:** Cytotoxic T Lymphocytes

**CXCR4**: C-X-C Chemokine Receptor type 4

**CYP2D6**: Cytochrome P450 2D6

**DAVID**: Database for annotation, visualization and integrated discovery

**DAXX**: Death Associate protein 6

**DC**: Dendritic Cells

**DMEM**: Dulbecco's Modified Eagle Medium

**DR4/DR5**: Death Receptor 4/ Death Receptor 5

**ECM:** Extracellular Matrix

**EGF**: Epidermal Growth Factor

**EGFR**: Epidermal Growth Factor Receptor

**LDA**: Limiting Dilution Assay

**EMT**: Epithelial-to-Mesenchymal Transition

**ERCC1/ERCC2**: Excision Repair Cross-Complementation group 1/Excision Repair Cross-Complementation group 2

**ESCs:** Embryonic Stem Cells

**FA**: Formic acid

**FACS**: Fluorescence-Activated Cell Sorting

**FAD:** Flavin Adenine Dinucleotide

**FBS:** Fetal Bovine Serum

**FDA:** Food and Drug Administration

**FDR**: False discovery rate

**Fluo$^-$**: Non-autofluorescent cells

**Fluo$^+$**: Autofluorescent cells

**FMN:** Flavin Adenine Mononucleotide

**FTC**: Fumitremorgin C

**FUBP1**: FUSE-Binding Protein 1

**GABARA1**: Gamma-Aminobutyric Acid, receptor alpha 1

**GA-MSCs:** Glioma Associated-MSCs

**GBM**: Glioblastoma

**G-CIMP**: Glioma CpG Island Methylator Phenotype

**GDFs**: Growth and Differentiation Factors

**GFAP:** Glial Fibrillary Acidic Protein

**GO**: Gene ontology

**GPX1**: Glutathione Peroxidase 1

**GSCs**: Glioblastoma Stem Cells

**GST**: Glutathione S-Transferases

**GWAS:** Genome-Wide Association Study

**HIF-1:** Hypoxia Inducible Factor 1

**HSV-tk:** Herpes Simplex Virus thymidine kinase

**HUCPVCs**: Human Umbilical Cord Perivascular Cells

**$IC_{50}$**: Half-maximal inhibitory concentration

**IDA**: Information-dependent acquisition

**IDH**: Isocitrate Dehydrogenase

**IFNRs:** IFN Receptors

**IFNα/β**: Interferon α/Interferon β

**IL**: Interleukin

**ISCT**: International Society for Cellular Therapy

**KEGG**: Kyoto encyclopedia of genes and genomes

**KPS:** Karnosfsky Performance Score

**KRAS**: KRAS proto-oncogene; formerly Kirsten rat sarcoma viral oncogene

**L1CAM:** L1 Cell Adhesion Molecule

**LeX**: Lewis-X Antigen

**LIF:** Leukemia Inhibitory Factor

**LIG1**: DNA Ligase 1

**LOH**: Loss of Heterozygosity

**MACS**: magnetic-Activated Cell Sorting

**MAPK**: Mitogen-Activated Protein Kinase

**MCP**: Monocyte Chemotactic Protein

**MDM2/MDM 4:** Murine Double Minute Genes 2/Murine Double Minute Genes 4

**MDR:** Multidrug Resistance

**MET:** hepatocyte growth factor receptor

**MGMT:** O6-Methylguanine-DNA Methyltransferase

**MLH1**: MutL Homolog 1

**MMP:** Matrix Metalloproteinases

**MDR:** Multidrug Resistance

**mRNA**: messenger RNA

**MRP1:** Multidrug Resistance Associated Protein 1

**MSCs:** Mesenchymal Stem Cells

**MSH2/MSH6:** MutS Homolog 2/MutS Homolog 6

**mTOR**: Mechanistic Target Of Rapamycin

**MVP**: Microvascular Proliferation

**NADP/NADPH**: Nicotinamide Adenine Dinucleotide Phosphate

**NEFL:** Neurofilament, light polypeptide

**NF1/NF2**: Neurofibromin 1/ Neurofibromin 2

**NK cells**: Natural Killer Cells

**NOS**: Not Otherwise Specified

**NOS1**: Nitric Oxide Synthase 1

**NPCs**: Neural Progenitor Cells

**NRP-2**: Neuropilin-2

**NSCs**: Neural Stem Cells

**NSG:** NOD.Cg-*Prkdc*$^{scid}$ *Il2rg*$^{tm1Wjl}$/SzJ

**NSPCs:** Neural Stem and Progenitor Cells

**NT-3**: Neurotrophin-3

**OD**: Odds Ratio

**OPCs**: Oligodendrocyte Precursor Cells

**OS**: Overall Survival

**OVs**: Oncolytic Viruses

**PAI-1**: Plasminogen Activator Inhibitor 1

**PARP1**: Poly (ADP-ribose) Polymerase 1

**PCR-RFLP**: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

**PCV:** Procarbazine, CCNU and Vincristine

**PDGF**: Platelet-Derived Growth Factor

**PDGF-A/PDGF-B/PDGF-C/PDGF-D:** Platelet-Derived Growth Factor A, B, C and D

**PDGFRα/PDGFRβ**: Platelet-Derived Growth Factor Receptor alpha and beta

**Pen/Strep**: Penicillin and Streptomycin

**PHLDB1**: Pleckstrin Homology-Like Domain, Family B, Member 1

**PI3K**: Phosphatidilinositol-3-Kinase

**PIK3CA:** Phosphatidylinositol 3-Kinase Catalytic subunit Alpha

**PIK3R1**: Phosphatidylinositol 3-Kinase Regulatory Subunit 1

**PMS2**: PostMeiotic Segregation increased 2

**PRC2**: Polycomb Repressive Complex 2

**PRKDC**: Protein Kinase, DNA-activated, Catalytic

**PTEN**: Phosphatase and Tensin homolog

**qRT-PCR:** quantitative-Reverse Transcriptase- Polymerase Chain Reaction

**RAF1**: Raf-1 proto-oncogene; formerly v-raf-1 murine leukemia viral

**RB**: Retinoblastoma

**RBF**: Riboflavin

**rCE**: rabbit Carboxylesterase Enzyme

**RNA:** Ribonucleic Acid

**RPMI**: Roswell Park Memorial Institute 1640

**RTEL1**: Regulator of Telomere Elongation Helicase 1

**RTK:** Receptor Tyrosine Kinase

**SCs:** Stem Cells

**SD:** Side Population

**SD**: Standard deviations

**SDF-1α:** Stromal cell-Derived factor 1 alpha

**Sema7A**: Semaphorin-7A

**SLC12A5:** Solute Carrier family 12 (potassium/chloride transporter), member 5

**SNP**: Single Nucleotide Polymorphism

**SOD2/SOD3:** Superoxide Dismutase 2/Superoxide Dismutase 3

**SSEA-1:** Stage-Specific Embryonic Antigen-1

**SYT1**: Synaptotagmin 1

**TA-MSCs:** Tumor Associated-MSCs

**TCA**: Trichloroacetic acid

**TCGA**: The Cancer Genome Atlas

**TCTP**: Translationally-Controlled Tumor Protein

**TERC**: Telomerase RNA Component

**TERT**: Telomerase Reverse Transcriptase

**TET**: Ten-Eleven Translocation enzymes

**TGF-β:** Transform growth factor- beta

**TGFβIp/ig-h3**: Transforming Growth Factor-Beta-Induced Protein/Ig-H3

**TICs**: Tumor Initiating Cells

**TIMP-1/TIMP-2:** Tissue Inhibitors Of Metalloproteinases

**TMZ**: Temozolomide

**TNF**: Tumor Necrosis Factor

**TP53:** Tumor Protein 53

**TPCs**: Tumor Progenitor Cells

**TRAIL**: Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand

**Treg**: T regulatory cells

**TSC1/TSC 2**: Tuberous Sclerosis 1/Tuberous Sclerosis 2

**UCB-MSCs:** Umbilical Cord Blood-Derived Mesenchymal Stem Cells

**Upar:** Urokinase plasminogen activator receptor

**VEGF:** Vascular Endothelial Growth Factor

**VEGFR:** Vascular Endothelial Growth Factor Receptor

**WHO:** World Health Organization

**WJ-MSCs**: Wharton’s Jelly Mesenchymal Stem Cells

**Wnt**: Wingless type mmtv integration site family

**XRCC1**: X-Ray Repair Cross Complementing 1

# Aims and Thesis Layout

# Aims and Thesis Layout

Gliomas, particularly glioblastomas (GBM), are one of the most devastating human cancers, presenting a high mortality rate and very poor patient outcomes. In the last decades this has not changed significantly, emphasizing the need for a better and more integrated understanding of its pathophysiology and a more rational design of novel therapeutic strategies that may, more efficiently, overcome the highly resistant nature of these tumors. The etiology of glioma remains largely undetermined being ionizing radiation the only risk factor firmly established; however, gliomas can also be caused by genetically inherited disorders and some genetic polymorphisms can modulate the risk for this type of tumors. Moreover, GBMs are highly heterogeneous tumors with a remarkable resistance to current therapies, which can partly be explained by the presence of glioblastoma stem cells (GSCs).

The **general aim** of this thesis is to better understand the molecular and cellular determinants that may impact on the pathophysiology of gliomas, with potential to affect the risk and prognosis of glioma patients. An emphasis is put on GBM, as this is the most common and aggressive glioma subtype. The thesis is organized in five chapters, each addressing a specific goal, as presented below.

**Chapter 1** presents a general introduction reviewing the current knowledge on glioma pathophysiology, aiming to prepare the reader for the work presented in the thesis. Briefly, this chapter summarizes the epidemiology, classification, molecular determinants, and treatment of malignant gliomas. An emphasis is given on GSCs as critical components of glioma pathophysiology, and on mesenchymal stem cells (MSCs) as novel putative tools for a stem-cell based therapy for glioma treatment.

**Chapter 2** focuses on the relevance of two single nucleotide polymorphisms (SNPs) of the *transforming growth factor-beta 1* (*TGF-β1*) gene on glioma risk and prognosis. Since *TGF-β1* genetic polymorphisms have been implicated in the susceptibility of several cancers, and TGF-β signaling pathway has been identified as a mediator on gliomagenesis (by stimulating tumor cell proliferation, invasion and angiogenesis), we studied the relevance of *TGF-β1* SNPs as biomarkers of glioma .This chapter is presented as a final research paper published in Tumor Biology (DOI 10.1007/s13277-015-3343-0).

**Chapter 3** presents a research work that identifies an intrinsic autofluorescent phenotype in GSCs that can be used as a biomarker, which is presently a topic of great interest in the field of cancer stem cells. In fact, GSCs have been associated with increased therapy resistance and tumorigenesis, making them critical therapeutic targets. While substantial progress has been made towards isolating GSCs, the currently-available GSCs markers lack full specificity and sensitivity, making the identification of new, more specific and reliable biomarkers of GSCs critical.

**Chapter 4** focuses on MSCs, and how they can affect glioma cells paracrinally via secreted molecules. This is critical as MSCs have been exploited as a potentially useful new stem cell-based therapy for cancer treatment. However, very few studies have evaluated the effect of MSCs secretome on GBM aggressiveness, reporting inconsistent findings. This chapter summarizes how MSCs' secretome modulates critical hallmark features of GBM, particularly on tumor cell viability, migration, proliferation, growth, and response to chemotherapy, while also characterize the full protein content of MSCs' secretome by proteomic analysis.

**Chapter 5** includes an overall discussion of the topics addressed in the thesis, highlighting the major contributions, limitations, and significance of our findings, integrating and discussing them at the light of current relevant literature. Finally, suggestions for relevant future directions to complement our research are presented, as well as a general conclusion.

# Chapter 1: General Introduction

# 1. General Introduction

## 1.1 Epidemiology and Classification of Glial Tumors

### 1.1.1 Epidemiology and Clinical Features of Gliomas

The central nervous system (CNS) encompasses several tumor subtypes that comprises benign to malignant entities, being the non-malignant tumors the most frequent (239,835 versus 117,023 malignant)[1]. CNS tumors are the most common cancer among children and adolescents (0-19 years of age) with an overall average annual age-adjusted incidence rate of 5.57 per 100,000. In adults (20+ years), the overall incidence rate is 28.57 per 100,000[1]. Regarding incidence rates by gender, CNS tumors are more frequent in females (58%; 206,565 tumors) than males (42%; 150,271 tumors)[1].

More than 250,000 new cases of primary malignant brain tumors are diagnosed annually, being the 17th most common cancer type worldwide[2]. United States of America (USA) white population, Northern Europe, and Israel are the regions presenting the highest reported rates of primary malignant brain tumors (11-20 per 100,000 habitants), while India and Philippines have the lowest rates (2-4 per 100,000 persons), which may be a consequence of differences in adequate health care and diagnosis practices, rather than geographic and genetic variations[3]. Among primary malignant brain tumors approximately 80% are gliomas[4]. In the USA, more than 19,000 new cases of glioma are diagnosed each year, with an age-adjusted average annual incidence of 6.6 per 100,000 persons[1, 5]. Males have higher incidence rates of glioma than females (1.3-fold), while gliomas are more common in non-Hispanic whites than African Americans[1, 5].

Gliomas constitute a broad class of neuroectodermal tumors believed to be originated from glial cells or stem/progenitor cells that upon malignant transformation develop glial characteristics[6, 7]. These tumors can appear anywhere in the CNS but occur primarily in the brain, preferably in the frontal, parietal, occipital, and temporal lobes combined (60.9%), and form a heterogeneous group of tumors with several histologic types and malignancy grades[5]. The classification of gliomas is performed according to the World Health Organization (WHO), which is based on their localization, histological features, degree of differentiation, grade of malignancy and, more recently, molecular features[4, 8].

Glioblastoma (GBM, WHO grade IV) is the most common type of glioma (55.1%), with an annual incidence of 3.2 per 100,000 persons[1]. The incidence of this tumor type increases with age, with rates highest in the 75 to 84 years old, being more common in older adults and less common in children. GBM is more common in males than females (1.6-fold) and is 2 times more prevalent in whites comparing with blacks[1]. Regarding survival rates, patients diagnosed with GBM present a low survival, with only 5.1% of patients being alive after 5 years post diagnosis[1]. The majority of GBM (~90%), arise *de novo* being designated primary GBM, while those that appear from a lower-grade lesion progression are designated secondary GBM.

Some general symptoms of gliomas include headache, confusion or a decline in brain function, memory loss, nausea or vomiting, personality changes or irritability, difficulty with balance, urinary incontinence, vision problems, speech difficulties and seizures. These neurological symptoms depend primarily on the CNS tumor location, however, the chance of a long recurrence-free survival is more closely associated with patient clinical history, including age and Karnofsky performance score (KPS), as well as, with the biology of the tumor[4].

### 1.1.2 Histological and Molecular Classification of Gliomas

Until recently, gliomas have been traditionally classified as astrocytomas, oligodendrogliomas, oligoastrocytomas (mixed), or ependymomas based on microscopic analysis (Table 1.1[4]). Additionally, the WHO classification assigns to each tumor a histologic grade that range from WHO grade I (lower grade) to WHO grade IV (higher grade[9]). Grade I gliomas are benign tumors presenting a low proliferative growing and are normally associated with a favorable prognosis. Grade II tumors are considered low grade gliomas presenting a slow growing and some malignant features such as diffuse infiltration. After surgical resection these tumors tend to progress to higher grade gliomas. Grades III and IV are considered high-grade malignant gliomas since they present features of high aggressiveness such as microvascular proliferation (MVP), nuclear atypia, necrosis and mitotic activity. From these, the most malignant gliomas are those of grade IV and present poor outcomes[4, 10, 11].

For several decades, histological classification was the “gold standard” for gliomas classification and forming the basis for patient treatment, however it is associated with considerable interobserver variability[12]. In the last two decades, several molecular studies demonstrated that within the same glioma entity extensive clinical and biologic variability occurred, suggesting that the genetic factors underlying this variation could be used as clinically relevant biomarkers, contributing to a more accurate classification of brain tumors[4, 9]. In fact, it was already demonstrated that molecular characteristics correlates better with the biology of gliomas than histological classification[13, 14].

**Table 1.1: Classification of gliomas according to WHO 2007 classification of central nervous system tumors [4].**

| Glioma type | Grade (WHO) | Glioma histologic subtype | Incidence (% of all brain tumors) |
|---|---|---|---|
| Astrocytoma | I | Pilocytic astrocytoma | 5-6% |
| | I | Subependymal giant cell astrocytoma | <1% |
| | II | Diffuse astrocytoma | 10-15% |
| | III | Anaplastic astrocytoma | 10-15% |
| | IV | Glioblastoma | 12-15% |
| Oligodendroglioma | II | Oligodendroglioma | 2.5% |
| | III | Anaplastic oligodendroglioma | 1.2% |
| Oligoastrocytoma | II | Oligoastrocytoma | 1.8% |
| | III | Anaplastic oligoastrocytoma | 1% |
| Ependymoma | I | Subependymoma | 0.7% |
| | I | Myxopapillary ependymoma | 0.3% |
| | II | Ependymoma | 4.7% |
| | III | Anaplastic ependymoma | 1% |

Therefore, recently both histological and molecular features of brain tumors were integrated in the new WHO classification of CNS tumors (Table 1.2[8]). This “integrated diagnosis” allow a more robust and quality-controlled assessment of clinically relevant diagnostic, prognostic, and predictive biomarkers. One of the major findings that validated the importance of gliomas’ molecular classification was the discovered of *isocitrate dehydrogenase* (*IDH*) *1* or *2*-mutation status. Indeed, it was established that the presence of *IDH* mutations distinguishes gliomas with distinct clinical behaviors and biologies[14-16]. For the purposes of this thesis, the major subtypes of glioma (astrocytomas and oligodendrogliomas) are further discussed throughout this chapter.

In adults, most glial tumors are diffuse gliomas that include the WHO grades II and III astrocytomas, the grades II and III oligodendrogliomas and the grade IV GBM, among other entities (Table 1.2[8]). These types of gliomas present a diffuse infiltrative growth within CNS parenchyma that is often further accompanied by aggregation of tumor cells around blood vessels, neurons (perineuronal satellitosis) and under pial membrane[17]. Additionally, diffuse gliomas tend to invade over large distances along myelinated fiber tracts that can crossing the corpus callosum into the opposite hemisphere (“butterfly glioma” pattern). Sometimes, a widespread diffuse glioma may have MVP and/or necrosis, as well as, multiple foci of high cellularity (multicentric or multifocal)[17].

**Table 1.2: Grading of diffuse astrocytic and oligodendroglial tumors according to WHO 2016 classification of central nervous system tumors[8].**

| Glioma histologic subtype | Grade (WHO) |
|---|---|
| Diffuse astrocytoma, *IDH* mutant | II |
| Gemistocytic astrocytoma, *IDH* mutant | II |
| Diffuse astrocytoma, *IDH* wildtype | II |
| Diffuse astrocytoma, NOS | II |
| | |
| Anaplastic astrocytoma, *IDH* mutant | III |
| Anaplastic astrocytoma, *IDH* wildtype | II |
| Anaplastic astrocytoma, NOS | III |
| | |
| Glioblastoma, *IDH* wildtype | IV |
| Giant cell glioblastoma | IV |
| Gliosarcoma | IV |
| Epithelioid glioblastoma | IV |
| Glioblastoma, *IDH* mutant | IV |
| Glioblastoma, NOS | IV |
| | |
| Diffuse midline glioma, *H3-K27M* mutant | IV |
| | |
| Oligodendroglioma, *IDH* mutant and *1p/19q* co-deleted | II |
| Oligodendroglioma, NOS | II |
| | |
| Anaplastic oligodendroglioma, *IDH* mutant and *1p/19q* co-deleted | III |
| Anaplastic oligodendroglioma, | III |
| | |
| Oligoastrocytoma, NOS | II |
| Anaplastic oligoastrocytoma, NOS | III |

### Histological classification

Traditionally, the evaluation of diffuse glioma subtypes is based on the similarity between non-neoplastic glial cells and tumor cells. Tumors showing hyperchromasia and nuclear irregularities are considered astrocytomas, whereas oligodendrogliomas usually have uniformly rounded nuclei[17]. Low grade oligodendrogliomas (WHO grade II) present uniform and round nuclei with a delicate chromatin, crisp nuclear membrane and very small nucleoli, while WHO grade III oligodendroglioma have increased pleomorphism, cell size, a more vesicular chromatin and prominent nucleoli, as well as may present perinuclear halo[17]. Additionally, histological features such as mitotic activity, necrosis, and MVP are used for grading of diffuse gliomas. WHO grade II diffuse gliomas, do not present malignant histological features such as necrosis, MVP and mitotic activity[18], while anaplastic high-grade gliomas (WHO grade III) are characterized histologically by increased mitotic activity, nuclear atypia and hypercellularity[19]. Finally, the defining histopathologic features for the diagnosis of GBM (WHO grade IV) are MVP and necrosis, nevertheless these tumors also present nuclear atypia, high mitotic rates, cellular pleomorphism, invasiveness and vascular thrombosis [4, 8].

### Molecular classification

Molecularly, diffuse astrocytic and oligodendroglial tumors are subdivided into several glioma subtypes which includes WHO grades II-IV *IDH*-wildtype, WHO grades II-IV *IDH*-mutant astrocytomas, *IDH*-mutant and short arm of chromosome 1 and the long arm of chromosome 19 (*1p/19q)*-co-deleted oligodendrogliomas of WHO grades II-III (Table 1.2 [8]). The hallmark genetic alteration of WHO grades II-III diffuse gliomas (astrocytoma and oligodendroglioma) is the mutation of *IDH1* or, less common, *IDH2*[16, 20, 21]. However, some alterations are different in astrocytic and oligodendroglial tumors. Mutations on *tumor protein 53* (*TP53*), and *alfa thalassemia/mental retardation syndrome X-linked* (*ATRX)* occur in WHO grades II and III astrocytomas, whereas whole-arm losses of *1p 19q* (*1p/19q* co-deletion) and *telomerase reverse transcriptase* (*TERT*) promoter mutation happen on oligodendrogliomas[13, 22]. In fact, *IDH*, *TP53*, and *ATRX* mutations are considered the defining molecular characteristics of astrocytic tumors, while *IDH* mutation, *1p/19q* co-deletion, and *TERT* promoter mutation is considered the genetic profile of oligodendrogliomas [13, 22] (Figure 1.1[9]).

WHO grade IV GBMs are divided into: GBM *IDH*-wildtype (~90%), GBM *IDH*-mutant (~10%) and GBM NOS (not otherwise specified), that corresponds to cases for which *IDH* mutation cannot be tested. Almost all *IDH*-wildtype GBMs are typically seen in patients over 55 years of age, and correspond to primary GBMs, while *IDH*-mutant GBMs correspond to secondary GBMs and preferential occur in young adults[8, 23]. In adults, *IDH*-wildtype GBMs frequently reveal homozygous deletion of the *cyclin-dependent kinase inhibitor 2A* and *2B* (*CDKN2A*/*p14ARF* and *CDKN2B*) loci on *9p21*, monosomy of chromosome 10, copy number gains on chromosome 7, and mutations in the promoter of *TERT* and in the *phosphatase and tensin homolog on chromosome 10* (*PTEN*) tumor suppressor gene[19]. Additionally, other mutations have been found in this GBM subtype, including mutations in the *neurofibromatosis* *type 1* (*NF1*), *TP53*, *phosphatidylinositol 3-kinase, catalytic, alpha* (*PIK3CA*), and *phosphatidylinositol 3-kinase regulatory subunit 1* (*PIK3R1*) genes[24]. Amplification of the *murine double minute genes* (*MDM2* and *MDM4*), *CDK4*, *CDK6*, and *epidermal growth factor receptor* (*EGFR*), *hepatocyte growth factor receptor* (*MET*), and *platelet-derived growth factor receptor A* (*PDGFRA*) genes was also observed[19].

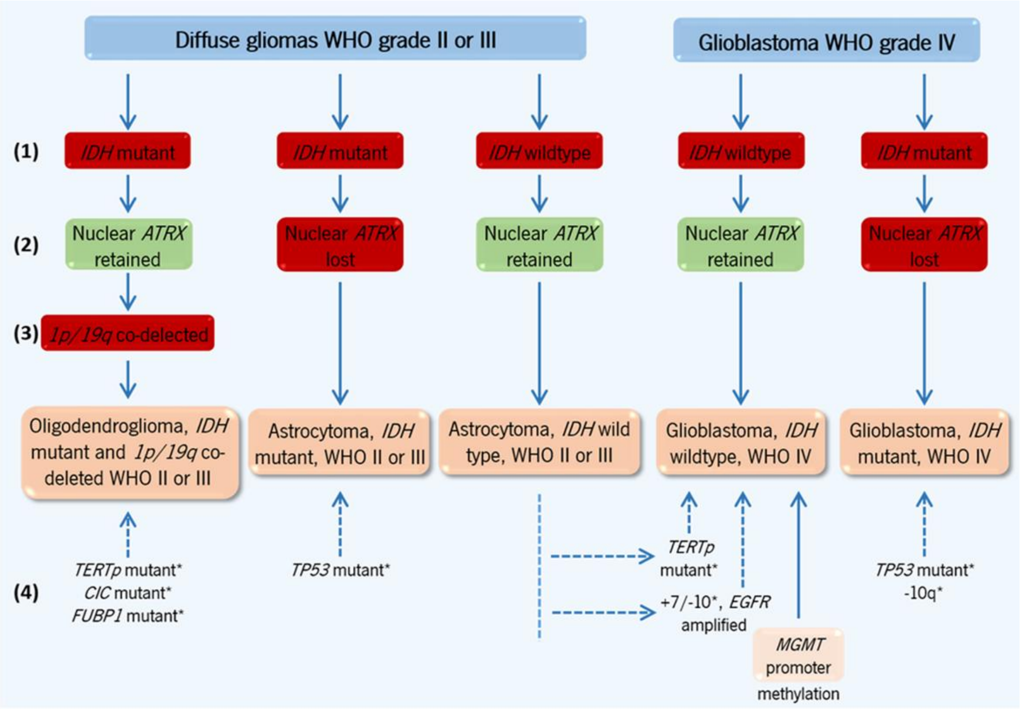

**Figure 1.1: Molecular diagnostics of diffuse gliomas.** The genetic alterations considered as diagnostic biomarkers, namely loss of nuclear *ATRX* expression, *H3-K27M* mutation, *IDH* mutation (mutation of *IDH1* or *IDH2*) and *1p/19q* co-deletion are shown, as well as the typical biomarker patterns for the most common diffuse glioma entities. Selected chromosomal and genetic alterations that may serve as additional diagnostic markers are also represented. *O6-methylguanine-DNA methyltransferase* (*MGMT*) promoter methylation does not represent a diagnostic marker but is of clinical importance as predictive marker for response to alkylating agent chemotherapy, in particular in elderly patients with *IDH*-wildtype GBM. **Abbreviations**: *TERTp* mutant, *TERT* promoter mutant; WHO II, III, or IV, WHO grade II, III, or IV. Results obtained by testing: (1) *IDH1* or *IDH2* mutations; (2) nuclear *ATRX* expression; (3) *1p/19q* co-deletion; (4) other markers (Adapted from [9]).

## 1.2 Determinants of Glioma Risk and Pathophysiology

### 1.2.1 Environmental and Genetic Risk Factors for Glioma

The main epidemiologic causes of glioma risk are advanced age, Caucasian race and male gender[25]. However, it is accepted that several environmental causes, such as ionizing radiation, allergies, tobacco smoking, alcohol consumption, diet, infectious agents, among others and genetic factors can be involved in the etiology of gliomas. Several studies have been evaluating the effect of those risk factors on glioma incidence, however most of the results were inconsistent and no clearly correlation was observed [5], highlighting that the etiology of gliomas is still unclear.

Regarding environmental risk factors, the only factor associated with increased glioma risk is ionizing radiation (Table 1.3[3]), which can induce DNA damage (both single- and double-strand breaks) that can cause genetic changes leading to cancer, including gliomas [26]. It has been shown that exposure to a high-dose of therapeutic radiation is the most firmly established environmental cause of glioma and that some genetic factors can influence the extent of risk from these exposures [18, 27-31]. Gliomas may appear as early as 7-9 years after irradiation[11]. In 1999, Salminen and colleagues showed that patients with brain tumors that were previously treated with radiation therapy developed more frequently secondary brain tumors[32]. Additionally, association studies between atomic bomb survivors and incidence of glioma were performed showing that survivors have higher incidence rates of glioma[33]. A higher relative risk of glioma was also observed in children that were treated with ionizing radiation for tinea capitis and skin hemangioma[34]. Moreover, several studies have already evaluated the association between glioma risk and the exposure of typical environmental risk factors, such as mobile phones[5, 35-37], electromagnetic fields[38, 39], diet (e.g., nitrosamine compounds, vitamin E; calcium intake)[40-46], infectious agents (e.g., influenza, chicken pox)[47-50], lifestyle behaviors (e.g., tobacco smoking, alcohol consumption)[44, 51], and allergies conditions (asthma, eczema, food allergies, hayfever)[52-58]. However, with the exception of allergies conditions, which has been associated with a reduced glioma risk [30, 52, 53, 55, 56, 58, 59], inconsistent associations, with one or more studies finding a positive association and others observing no association, have been reported (Table 1.3[3]).

For the above-mentioned reasons, it is important to further investigate which environmental risk factors are associated with glioma risk by performing large-scale studies with better assessment of exposure, together with the analysis of genetic factors that may influence the effects of such exposure.

Heritable genetic contribution to glioma has been suggested by studies of genetic syndromes, familial aggregation and linkage[60, 61]. The familial tumor syndrome most frequently associated with glioma is Li-Fraumeni syndrome that is caused by a constitutive loss-of-function mutation in *TP53*. Nevertheless, other rare Mendelian disorders are associated with increased glioma risk, such as Lynch and melanoma-neural system tumor syndromes, Neurofibromatosis 1 and Neurofibromatosis 2 and tuberous sclerosis (Table 1.4 [3]). Since only a minority of glioma cases are caused by ionizing radiation effects and inherited disorders, segregation analyses have found that genetic risk factors for glioma are best explained with a polygenic model. Therefore, several studies assessing genetic polymorphisms thought to be involved in glioma susceptibility were performed.

**Table 1.3: Environmental risk factors studied as possible glioma risk factors[3].**

| Environmental factor | Association |
|---|---|
| **Ionizing radiation** | + |
| **Mobile phone use** | x |
| **Electromagnetic fields** | x |
| **Tobacco smoking** | x |
| **Alcohol consumption** | x |
| **Nitrosamine compounds** | x |
| **Allergies conditions** | - |
| **Chicken pox** | x |

**Abbreviations:** +, association with increased glioma risk; -, association with decreased glioma risk; x, no consistent associations.

Single nucleotide polymorphisms (SNPs) are the most frequent type of genetic variation in the human genome[62], and can impact gene expression, function, phenotypes and diseases [63]. SNPs contribute deeply to cancer susceptibility, and their study has proven fundamental in defining disease candidate gene regions. Some of the most frequently studied polymorphisms are in genes involved in DNA repair (a mechanism extremely important in the preservation of genomic integrity), cell cycle/apoptosis (deregulation of cell proliferation and apoptosis is considered a hallmark of human tumors, including glioma), cancer metabolism (important in cellular detoxification process), growth pathways (deregulation of growth signaling pathways is another hallmark of gliomas), among others. Regarding DNA repair mechanisms, several genes, including *MGMT* (O-6-methylguanine-DNA methyltransferase), *protein kinase, DNA-activated, catalytic* (*PRKDC*), *excision repair cross-complementation group* (*ERCC1*) *1 and 2*, *X-Ray Repair Cross Complementing 1* (*XRCC1*), *APEX1*, *TP53*, *Poly (ADP-ribose) polymerase 1* (*PARP1*), and *DNA ligase 1* (*LIG1*) have been studied in the susceptibility to develop glioma[26, 64-67]. Additionally, genes involved in cell cycle/apoptosis, such as *caspase 8*, *cyclin D1* (*CCND1*), *cyclin H* (*CCNH*) *MDM2*[68, 69], in cancer metabolism, including *Glutathione S-transferases* (*GST*), *Cytochrome P450 2D6* (*CYP2D6*), *Superoxide dismutase* (*SOD*) 2 and 3, *glutathione peroxidase 1* (*GPX1*), and *nitric oxide synthase 1* (*NOS1*)[70-72], and growth pathways, including *epidermal growth factor* (*EGF*) and *EGFR*[73, 74], were also investigated in glioma risk.

**Table 1.4: Hereditary cancer syndromes associated with increased risk of glioma [3].**

| **Gene** | **Syndrome** | **Features** | **Associated Gliomas** |
|---|---|---|---|
| *CDKN2A* | Melanoma-neural system tumor syndrome | Predisposition to melanoma and astrocytic tumors | Astrocytoma |
| *IDH1*/*IDH2* | Ollier disease/ Maffucci syndrome | Intraosseous benign cartilaginous tumors, cancer predisposition | Glioma |
| *MSH2*, *MLH1*, *MSH6*, *PMS2* | Lynch syndrome | Gastrointestinal, endometrial, and other cancers | Glioblastoma, astrocytoma |
| *NF1* | Neurofibromatosis 1 | Neurofibromas, schwannomas, Café-au-lait macules | Astrocytoma, optic nerve glioma |
| *NF2* | Neurofibromatosis 2 | Acoustic neuromas, meningiomas, neurofibromas | Spinal ependymoma |
| *POT1* | Melanoma-oligodendroglioma susceptibility syndrome | Predisposition to melanoma and oligodendroglial tumors | Oligodendroglioma and mixed oligoastrocytoma |
| *TP53* | Li–Fraumeni syndrome | Numerous cancers, especially breast, brain, and soft-tissue sarcoma | Glioblastoma, astrocytoma, choroid plexus tumor |
| *TSC1*, *TSC2* | Tuberous sclerosis | Multisystem nonmalignant tumors | Subependymal giant cell astrocytoma |

**Abbreviations:** *MLH1*, mutL homolog 1; *MSH2*/*MSH6*, mutS homolog 2/6; *NF1*/*NF2*, neurofibromin 1/2; *PMS2*, postmeiotic segregation increased 2; *TSC1*/*TSC2*, tuberous sclerosis ½ (Adapted from [5]).

These case-control studies only assessed a limited set of genetic polymorphisms, and a robustly replicated glioma risk loci was not found from those candidate genes. Additionally, inconsistent associations were found. Therefore, and taking advantage of recent technology that allows for a rapid whole genome sequencing, some genome-wide association studies (GWASs) of glioma patients were performed[75-80]. In a GWASs, healthy controls and individuals with the disease of interest are genotyped at hundreds of thousands of SNPs to discover inherited variants, which are significantly more common in those with disease than in those without[80]. The GWASs performed with glioma patients have identified 10 independently significant SNPs associations located in eight gene regions, which includes regions near to *CCDC26* (*Coiled-*

*Coil Domain Containing 26*) *CDKN2B*, *EGFR*, *Pleckstrin Homology-Like Domain, Family B, Member 1* (*PHLDB1*), *Regulator of Telomere Elongation Helicase* (*RTEL1*), *Telomerase RNA Component* (*TERC*), *TERT*, and *TP53* (Table 1.5[3]). These germline SNPs (also called glioma risk loci, risk alleles, or risk variants) were found to be more frequent in glioma patients than controls, indicating that persons inheriting one of these variants have an increased glioma risk by 20-40% when compared with a person who did not inherit that variant[81]. Some of these risk variants have previously been identified in glioma-associated hereditary cancer syndromes (i.e., *CDKN2B*, *TP53*) and glioma tumor studies (i.e., *CDKN2B*, *EGFR*, *TP53,*), while others are located in or near genes or chromosomal regions that were not been previously associated with glioma (i.e., *CCDC26*, and *PHLDB1*, of unknown function; and *RTEL1*, *TERC*, *TERT*, involved in telomere maintenance). These risk variants are not within the exonic portions of these genes, suggesting that inherited differences in gene regulation confer risk for glioma at these loci [3]. Four of these variants (*EGFR*, *RTEL1*, *TERT*, *TP53*) were associated with an increased risk of all glioma grades[80-82], while the other four regions contain variants associated with increased risk for specific glioma grades, histologies, or molecular subtypes (*CCDC26*, *CDKN2B*, *PHLDB1*, *TERC*)[80, 82-84]. SNPs in *CCDC26* (chromosome 8q24) have an increased risk of *IDH*-mutated astrocytomas and also of oligodendrogliomas, regardless of *IDH* mutation status[83]. SNPs near *CDKN2B* (chromosome 9) increase risk of astrocytomas, regardless of grade, but are not associated with risk of oligodendrogliomas[80]. Finally, SNPs in *PHLDB1* increase risk of *IDH*-mutated gliomas, regardless of grade or histology[84].

Important progress has been made in the identification of potential risk factors for glioma (ionizing radiation, and heritable genetic factors) although more studies are necessary. The new molecular tools, as well as the use of larger groups of patients, will probably provide the discovery of new inherent risk variants, which altogether can contribute to classify gliomas into more homogeneous subgroups concerning etiology. With this future analysis, the potential interaction between somatic alterations, inherited genetic variants, and environmental risk factors can be widely evaluated, leading to a further understanding in the process of gliomagenesis.

**Table 1.5: Hereditary variants associated with risk of glioma from GWASs[3].**

| Gene | SNP (risk allele) | Hypothesized function | Associated Glioma |
|---|---|---|---|
| ***CCDC26*** | rs55705857 (G) | Undetermined | Oligodendroglial tumors, IDH-mutated astrocytomas |
| ***CDKN2B*** | rs1412829 (G) | Increased ANRIL expression | Astrocytoma II–IV |
| ***EGFR*** | rs2252586 (A) | Undetermined | Astrocytoma III–IV |
| ***EGFR*** | rs11979158 (A) | Undetermined | Astrocytoma III–IV |
| ***RTEL1*** | rs6010620 (G) | Alteration of RTEL1-PCNA interaction domain | All glioma subtypes |
| ***RTEL1*** | rs4809324 (C) | Increased telomere length/telomerase activity | Astrocytoma III–IV |
| ***PHLDB1*** | rs498872 (A) | Undetermined | IDH-mutated glioma |
| ***TERC*** | rs1920116 (G) | Increased telomere length/telomerase activity | Astrocytoma III–IV |
| ***TERT*** | rs2736100 (C) | Increased telomere length/telomerase activity | All glioma subtypes |
| ***TP53*** | rs78378222 (C) | Alteration of TP53 polyadenylation signal | All glioma subtypes |

### 1.2.2 Molecular alterations/biomarkers on Glioma Pathophysiology

Several recent studies have tried to characterize the complex biology of gliomas, focusing on molecular analysis of tumors with apparently similar pathological features[85]. Integrated analyses on mutational data, DNA methylation and copy number, mRNA, microRNA and protein expression information were able to identify subgroups of gliomas that were more precisely defined than by histologic analysis[86-87]. Additionally, it was already demonstrated that in subsamples of the same GBM patient there is a genetic diversity, and more importantly that single GBM cells presented different patterns of genetic alterations[88-90]. Several studies have divided GBMs into multiple molecular classes, opening a new area of research into molecular markers of GBM[91-93]. In this section, the most relevant molecular markers of glioma, particularly in GBM, will be discussed.

#### Isocitrate dehydrogenase (IDH)

IDH enzymes catalyze the oxidative carboxylation of isocitrate α-ketoglutarate (α -KG), resulting in the reduction of nicotinamide adenine dinucleotide phosphate (NADP) to NADPH. IDH1 and 2 are encoded by the *IDH1* and *IDH2* genes, respectively[94]. In the last decade, the discovered of somatic mutation in *IDH1* and *IDH2* genes, in a subset of GBMs, was probably the greatest discovery in the molecular understanding of gliomas[15]. The authors showed that

*IDH* mutations occurred mostly in younger patients with secondary GBMs and were significantly associated with an increase in overall survival (OS)[15]. Subsequently, studies showed that mutations in *IDH1* or *IDH2* were mutually exclusive and that mutations in these genes consist of single amino acid substitutions. *IDH1* mutation (R132H) is the most common mutation of gliomas, accounting for about 90% of the mutated gliomas[15, 16, 21, 95-98]. Moreover, Christensen and colleagues demonstrated that the presence of *IDH1* or *IDH2* mutation was significantly associated with better survival of glioma patients, independently of patients' age, sex, and grade-specific histology[99]. Globally, mutations on *IDH1* and *IDH2* genes are more frequently found in WHO grade IV secondary GBM and WHO grade II and III of young adults (70%)[15, 21, 98]. Contrarily, pediatric diffuse gliomas or primary GBMs rarely present *IDH* mutations[15, 21, 100].

Some mechanisms for the tumorigenic potential of mutant IDH proteins have been suggested. *IDH1* mutation and its inactivation, activate hypoxia inducible factor 1 (HIF-1) pathways that are important in the inhibition of apoptosis, tumor growth, and cell survival under hypoxic conditions[101]. Additionally, Xu and colleagues have shown that *IDH1/2* mutations convert α-KG to 2-hydroxyglutarate (2-HG), which in turn inhibits α-KG-dependent dioxygenases, including members of the TET family of 5-methylcytosine (5mC) hydroxylases[102]. Inhibition of these enzymes can contribute to gliomagenesis since there is an increase on DNA and histone methylation, which can lead to aberrant methylation (hypermethylation) of multiple cytosine-phosphate-guanine (CpG) dinucleotide-rich islands across the genome, a characteristic profile designated as glioma CpG island methylator phenotype (GCIMP) [87, 99, 103-106].

*IDH* mutations occur early on gliomagenesis and possible represent the initiating somatic aberration in the vast majority of WHO grades II and III diffuse gliomas; nonetheless, these mutations do not appear to be sufficient for tumor growth induction[107] In malignant glioma patients, *IDH* mutation is associated with better response to temozolomide (TMZ) and adjuvant radiation and longer OS[22, 96, 97, 108-114].

Recent studies demonstrated that inhibitors of mutant *IDH1* promoted differentiation and inhibition of tumor growth of *IDH1* mutant glioma cells[115, 116]. Similarly, in immunized mice models of intracranial glioma, immunotargeting of mutant *IDH1* have shown antitumor immunity, tumor regression and prolonged survival[117, 118].

### Alpha-thalasemia mental retardation syndrome X-linked (ATRX)

ATRX is a DNA helicase and a chromatin remodeling protein. ATRX incorporates H3.3 histone proteins into the telomeric regions of chromosomes in collaboration with histone chaperone death associate protein 6 (DAXX)[119]. Mutations in *ATRX* gene result in the loss of protein function, which lead to abnormal telomeres, and are associated with an alternative lengthening of telomeres (ALT) phenotype, along with more widespread genomic destabilization[120, 121]. In gliomas, *ATRX* mutations have been associated with telomere maintenance and lengthening leading to evade apoptosis, and immortality of glioma cells[121-125].

Mutations in *ATRX* gene are less frequent in oligodendrogliomas and primary GBMs (4-20%). In contrast, these mutations are much more common in WHO grades II and III astrocytomas (> 60%) and in secondary GBMs (~57%)[123]. These mutations are more frequent in tumors that have *IDH* and *TP53* mutations, but are mutually exclusive with *1p/19q* co-deletion [123-125]. Patients with astrocytic tumors with loss of *ATRX* presented a significant better prognosis than those expressing this gene and simultaneously had *IDH* mutation[126].

### Telomerase reverse transcriptase (TERT)

Human telomerase is inactive in most adult cells being only active in the embryonic state or in high proliferative somatic cells. In the case of cancer cells, it is normal to observe a reactivation of telomerase. TERT, the catalytic subunit of the telomerase complex, is involved in telomere maintenance, by adding nucleotides to the telomeres. Mutations in the promoter region of *TERT* at positions 228 and 250 (C228T and C250T) increase the expression of telomerase[127]. In gliomas, the discovery of these mutations provided a biomarker for prognostication of brain tumors, being associated with a poor prognosis[127].

Activating mutations in *TERT* promoter occurred in a large percentage of primary GBM (54-83%) and oligodendrogliomas (77% of WHO II and III oligodendrogliomas and 82% of *1p/19q* co-deleted tumors), but were rare events in WHO grades II and III astrocytomas (26%) and secondary GBMs (5%)[128, 129]. These *TERT* mutations were inversely associated with tumors presetting *ATRX* and *IDH* mutations[129, 130], but were positively correlated with *EGFR* amplification. Importantly, mutation on *TERT* promoter has been associated with poor overall survival in GBM patients, particularly when associated with *EGFR* amplifications[130, 131].

### 1p/19q co-deletion

Co-deletion of *1p/19q* is an early genetic event and has been associated with tumors of the oligodendroglial lineage[132]. *1p/19q* co-deletion results from an unbalanced translocation involving the centromeric regions of 1p and 19q. So far, the role that this co-deletion has in tumorigenesis is not clear, however, recent studies, in a subset of oligodendroglial tumors, have identified mutations in two potential tumor suppressor genes, FUSE-binding protein 1 (*FUBP1*) in chromosome 1p and homolog of the Drosophila gene capicua (*CIC*) on chromosome 19, that may explain this question[133, 134].

Gliomas that harbor this co-deletion present better prognosis, since these patients have good response to chemotherapy and longer survival compared to similar histologic grade, and malignant progression to GBM is rare[135, 136]. Moreover, recent studies have found a strong correlation between *1p/19q* co-deletion and *IDH* mutation[114, 130, 133, 137].

### Epidermal growth factor receptor (EGFR)

EGFR is a transmembrane receptor tyrosine kinase that is frequently amplified in GBMs (40%), and less frequently in anaplastic astrocytomas (5-10%)[24]. *EGFR* amplification is highly frequent in primary GBMs[138]. Approximately 50% of GBMs with *EGFR* amplification are mutated in *EGFRvIII* form[139-141]. *EGFRvIII* is the most common *EGFR* mutation in GBM, being characterized by in-frame deletion of exons 2-7, which encode the extracellular surface of the protein that results in a truncated transmembrane receptor with constitutive activity. Both *EGFR* amplification and the *EGFRvIII* mutant are mutually exclusive with *IDH* mutations.

Tumors presenting both forms, *EGFR* amplification and *EGFRvIII* overexpression, were associated with GBM patients' poor prognosis. Nevertheless, it was shown that the prognostic value of *EGFR* amplification depends on GBM patient's age. Younger patients presenting *EFGR* overexpression and *TP53* wildtype presented a worse prognosis compared to older patients, indicating a relationship between age, *EGFR* and *TP53*[142]. Mechanistically, the constitutive activation of *EGFR* is associated with cell survival, growth, invasion, tumorigenicity and, radio- and chemo-resistance[143, 144]. In the last years, several therapies anti-EGFR (antibodies and small molecules inhibitors) have been developed, however none of these therapies were sufficiently effective.

### O6-methylguanine-DNA methyltransferase (MGMT)

One of the most clinically relevant DNA methylation in GBMs is in the promoter of *MGMT*. MGMT is a ubiquitously expressed nuclear enzyme that removes alkyl groups from the O6-position of O6methylguanine. This process interferes with the effect of TMZ (alkylating chemotherapeutic agent) since this DNA repair protein removes the alkyl groups induced by TMZ, leading to therapy resistance [145-147]. Hypermethylation of *MGMT* promoter causes gene silencing which interferes with DNA repair and increases TMZ sensitivity whereas an unmethylated promoter of *MGMT* lead to gene expression and consequently to elevated levels of the repair enzyme resulting in chemotherapy resistance [147].

Approximately 50% of primary GBMs present methylation of *MGMT* promoter[148-150]. However, this phenomenon is associated with *IDH1/2* mutant tumors because of which it is more common in secondary (75%) comparing with primary GBMs (36%)[96]. *MGMT* promoter methylation has prognostic and predictive significance in GBM patients, being associated with better OS independently of treatment[147, 151, 152]. Additionally, it is associated with better response to TMZ combined with radiotherapy (RT), improving progression free survival (PFS) and OS with combined treatment when compared with RT alone[147, 152-154]. Transcriptional silencing of the *MGMT* gene due to promoter hypermethylation occurs almost invariably in *IDH* mutant and *G-CIMP* positive diffuse gliomas, as opposed to approximately 50% of *G-CIMP* negative, *IDH* wildtype diffuse gliomas[130, 155].

### Transcriptional subtypes of GBM

Over the last two decades, the extensive use of DNA microarray technology proved to be a powerful tool providing new ways for tumor classification that can be used in diagnosis, prognostication and prediction. Early studies in malignant glioma verified that transcriptional signatures efficiently distinguish lower-grade tumors from GBM, as well as identified several genes whose expression levels correlate with prognosis[156-161]. Subsequently, some studies found that specific expression profiles could strongly discriminate primary from secondary GBMs[162-164]. Phillips and colleagues, in WHO grades II and III diffuse gliomas, examined differential expression of markers associated with clinical outcome and identified three major subclasses of GBM: proneural, mesenchymal and proliferative[92]. The proneural subtype was shown to be associated with a better prognosis, as well as to the expression of genes with normal brain and neurogenic processes. The other two subtypes, mesenchymal and proliferative, were associated with poor prognosis and showed activation of gene expression related to cell proliferation or angiogenesis, respectively[92]. In 2009, Verhaak *et al*, with a sample set of 200 GBMs from TCGA, employed unsupervised clustering of global transcriptional data. This analysis divided GBMs into four molecular subclasses: classical, mesenchymal, proneural and neural[93]. Classical, mesenchymal and proneural tumors were strongly associated with genomic abnormalities in *EGFR*, *NF1*, and *PDGFRA* and *IDH1* or *IDH2*, respectively. Regarding the neural subtype, this tumor type was characterized by the expression of neuron markers, such as *gamma-aminobutyric acid* (*GABA*) *A receptor, alpha 1* (*GABRA1*), *neurofilament, light* *polypeptide* (*NEFL*), synaptotagmin *1* (*SYT1*), and *solute carrier family 12* (*potassium/chloride transporter*), *member 5* (*SLC12A5*)[93]. Moreover, gene signatures revealed that proneural tumors correlated best with oligodendrocytes, neural tumors with mature neurons, and classical and mesenchymal tumors with astrocytes [93]. Regarding the response to aggressive therapy, the subtype that most improved with this therapeutic regime, was the classical, and no benefit was observed for proneural subtype[93].

## 1.3 Cell Biology of Glioma

### 1.3.1 Theories on Cell of origin of Glioma

The origin of gliomas is still a controversial subject. Nevertheless, it is accepted that all human malignant neoplasms arise from a series of molecular alterations that begin in a few numbers of cells, or even in a single. Currently, two distinctive models have been proposed for the origin of glioma: clonal model and cancer stem cell model[165, 166].

In 1976, Nowell and co-workers postulated that cancer was an evolutionary process, where tumors had diverse genetically and phenotypically cell subpopulations (clonal model). In this model, genetic or epigenetic mutations appear randomly and any new phenotypes are subjected to the pressure of natural selection, with the best adapted able to expand and proliferate [167]. This variability would become important when environmental changes occur, such as those induced by chemo- or radio-therapy, when the previous acquisition of a resistant phenotype would allow a minor population to survive, expand, and become dominant [167]. Regarding gliomas, it is hypothesized that these tumors have origin in differentiated mature glial cells (e.g., astrocytes or oligodendrocytes) that suffer a dedifferentiation after the primary alteration, during the carcinogenic process (Figure 1.2[168]).

Lately, the cancer stem cell (CSC) theory has become a widely accepted model of cancer initiation and progression. This model emphasizes the importance in cancer of a subset of cells capable of generating other cell types in a unidirectional manner [169, 170]. This theory postulates a hierarchical organization in which a tumor is generated from cells with stem cell characteristics, known as CSCs. By asymmetric division, these cells will maintain their population and, at the same time, generate more differentiated cells with limited proliferation that constitute the tumor bulk, while CSCs will remain as a small subpopulation. In this view, heterogeneity is the dualistic nature of CSCs and non-CSCs with various degrees of differentiation, regardless of their genetic background [171] presumably determined by epigenetic changes [165]. In the case of glial tumors, the CSC hypothesis postulates that these tumors are originated by an alteration on neural stem cells (NSCs) or neural progenitor cells (NPCs) (Figure 1.2[168])[166, 172, 173].

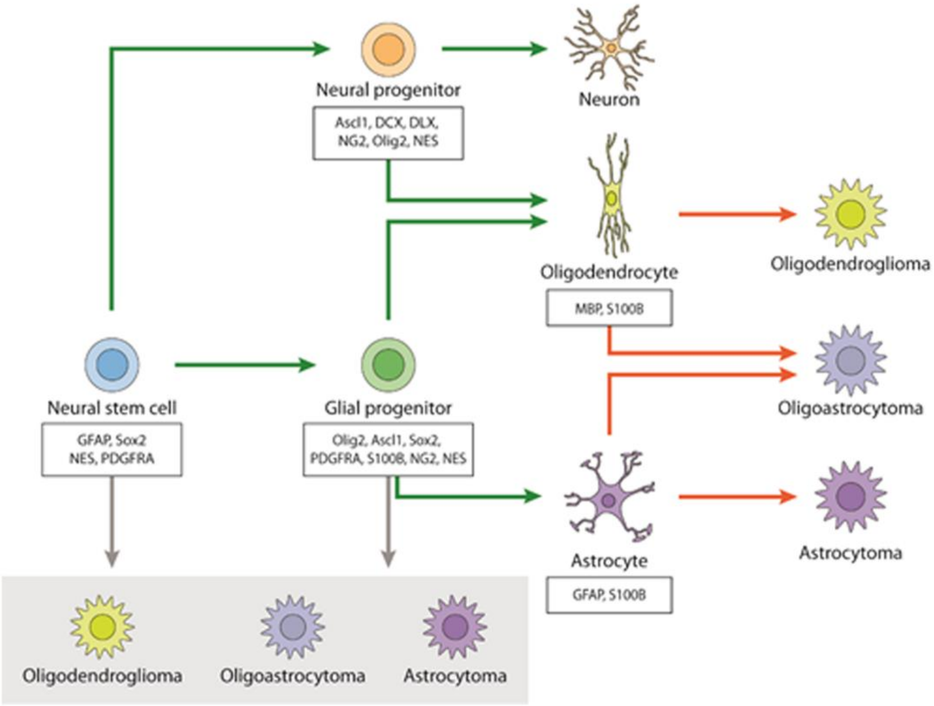

**Figure 1.2**: **Schematic representation of the differentiation process of neural stem cells into different cell lineages of the CNS and putative cells of origin of gliomas.** Protein markers for neural stem cells, progenitor cells, and differentiated cells are indicated in boxes. The normal differentiation process (green arrows) originates three main types of cells in the mature CNS, including neurons and glial cells (particularly oligodendrocytes and astrocytes; ependymal cells are not represented). The most classical hypothesis on the origin of glioma cells is represented by orange arrows (differentiated glial cells are malignantly transformed through a dedifferentiation process). The most recent hypothesis postulating that gliomas originate from the direct transformation of neural stem cells or glial progenitor cells is represented by grey arrows[168].

Although clonal evolution and CSC models have been considered as mutually exclusive, both models could be complementary since intraclonal heterogeneity has been observed in tumors in which CSCs were identified. Moreover, heterogeneity can be also generated by cell plasticity in response to microenvironment cues, such as blood vessel density, differences in oxygen pressure and composition of extracellular matrix. These differences will affect tumor cells and may be a cause of genetic and phenotypic changes observed in tumor cells. Therefore, and considering this view, the CSC model can be updated with the concept of various degrees of “stemness” and/or tumorigenic potential, determined either by stochastic events or microenviromental cues[174, 175].

Even though the CSC theory has been widely accepted, the origin of CSCs remains a mystery. Two distinct hypotheses are considered: i) CSC has origin in a normal stem cell or progenitor cell that undergoes specific genetic aberrations; ii) de-differentiation of differentiated cells in the early tumor occur to form CSCs. Stem cells produce transient cells, which in turn generate lineage-restricted progeny that become the differentiated effector cells (Figure 1.3). The pools of neural stem and progenitor cells (NSPCs) differ in location during development, suggesting that different cellular hierarchies may be co-opted by brain tumors[176]. In fact, normal stem cells or progenitor cells could be ideal targets for malignant transformation since they represent the most primitive cells, live longer, and typically re-enter cell division to replace the pool of both stem cells and differentiated progenies. Therefore, in theory, these stem/progenitor cells could accumulate sequential genetic or epigenetic mutations and initiate oncogenesis.

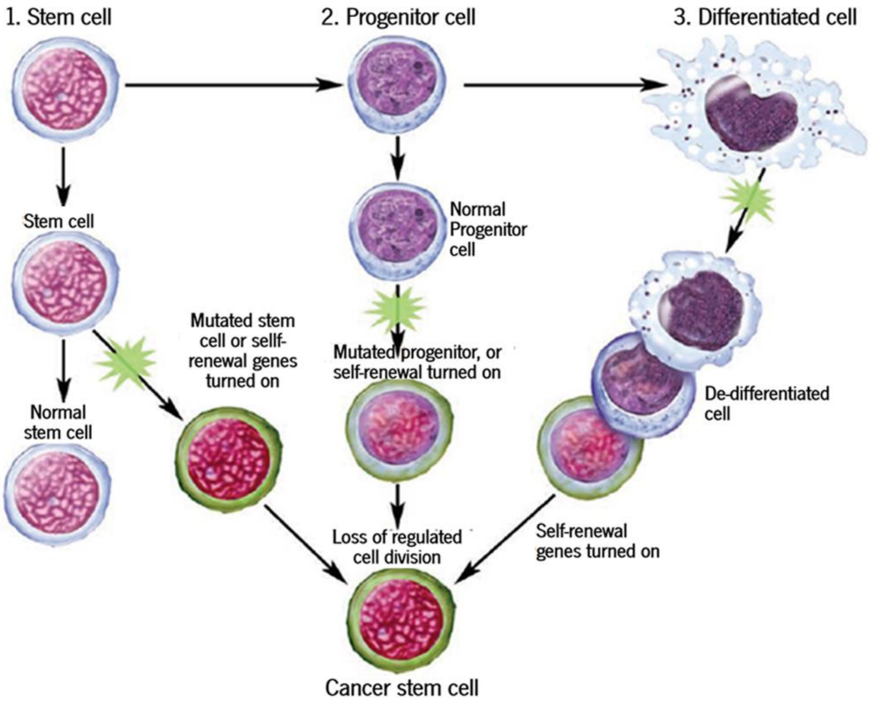

**Figure 1.3: Hypotheses of how a cancer stem cell may arise**. (1) A stem cell undergoes a mutation, (2) A progenitor cell undergoes two or more mutations, or (3) A fully differentiated cell undergoes several mutations that drive it back to a stem-like state. In all 3 scenarios, the resultant cancer stem cell has lost the ability to regulate its own cell division (Adapted from[177]).

The existence of a cell of origin (cell type that is uniquely susceptible to particular oncogenic mutation(s))[178] for CNS tumors has been explored using several transgenic animal models. These models have into consideration that NSPCs in the brain are the primary cellular targets for gliomagenesis[179]. These animal models use NSPC-related cell promoters, as nestin and glial fibrillary acidic protein (GFAP), to inactivate tumor suppressors (i.e., PTEN or p53) or drive oncogene expression (i.e., activated Ras) in specific cellular compartments. This process was effective in initiating cellular transformation and driving oncogenesis[180-184]. Moreover, in gliomas, differentiated cells in the CNS (neurons and astrocytes) demonstrated the ability to initiate tumorigenesis upon oncogenic transformation[185]. Additionally, several studies demonstrated that oligodendrocyte precursor cells (OPCs) can be the cell of origin for malignant gliomas, since these cells are susceptible to transformation by a wide range of mutations often found in human gliomas, such as *PTEN*, *Nf1*, *Ras sarcoma* (*Ras*) and *p53*[7].

These studies demonstrated that any cell in the brain can serve as a cell of origin for CNS tumors, and emphasize the importance of the inter-conversion between CSCs and differentiated cancer cells for tumor initiation and maintenance [7]. Therefore, to develop effective anticancer therapies, it is important to elucidate the molecular mechanisms behind this plastic behavior as well as to explore how conventional chemo- and radio-therapies can influence this process.

### 1.3.2 Oncogenic Signaling Pathways

GBM presents several different genetic and molecular alterations that lead to modifications of several major signaling pathways resulting in glioma growth and progression[186, 187]. It is well accepted that several signaling pathways such as, growth factor receptor tyrosine kinase (RTK)–triggered pathways, including the Ras pathway, the phosphatidylinositol 3-kinase (PI3K)/PTEN/AKT, transform growth factor- beta (TGF-β), retinoblastoma (RB)/CDKN2A-p16INK4a, and the TP53/MDM2/MDM 4/CDKN2A-p14ARF, are involved in gliomagenesis. Complex interactions among these pathways occur, which potentially contribute to the initiation and transformation of GBM (Figure 1.4[164]) [188].

#### Growth Factor RTK's Pathways

Both PDGF and EGF play an important role in normal homeostasis and gliomagenesis, by affecting cell proliferation, differentiation and metabolism, through activation of complex intracellular cascades modulated by G-protein–coupled receptors. The PDGF family comprises four different ligands (PDGF-A, PDGF-B, PDGF-C, and PDGF-D) that signal through the PDGF receptor (PDGFR) α and PDGFRβ[189]. The high expression of PDGFR suggests that these RTK-signaling pathways are critical targets in gliomagenesis [190]. Both the PDGF ligands and receptors are often co-expressed in glioma cell lines and primary GBM tissues that may contribute to tumor formation and progression. Since co-expression of PDGF and PDGFR has been detected in astrocytomas of all grades, PDGF autocrine signaling may be considered as an early event.

EGFR and its ligands have been associated with gliomagenesis, and are frequently overexpressed. EGFR is a transmembrane glycoprotein that operates as a RTK. In GBM cells, EGFR signaling may be activated in a ligand dependent or independent way, through overexpression of both the ligand(s) and the receptor. The oncogenic properties of EGFR are associated with a constitutive and uncontrolled increase in its phosphorylation (catalytic) activity. When its ligand bound to EGFR stimulates activation of signal transduction pathways involved in cell survival, proliferation, and differentiation. Additionally, *EGFR* amplification and/or its mutation leads to a constitutive activation in the absence of ligand[191, 192]. *EGFR* mutations or rearrangements are frequently found in GBM. The most frequent (30%) is variant 3 (*EGFRvII*I), which leads to a constitutive activation of RTK's pathway[193].

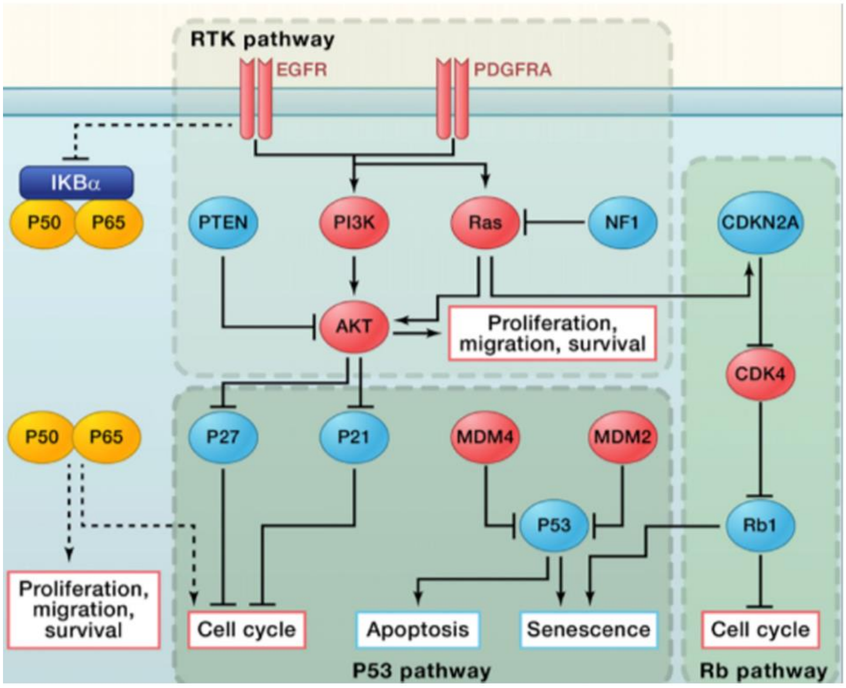

**Figure 1.4: Main Signaling Pathways in Glioma Tumorigenesis.** The receptor tyrosine kinase (RTK), p53, and Rb pathways are the core signaling pathways in gliomagenesis. In red are indicated oncogenes that are either overexpressed or amplified in GBM samples, and in blue are designated tumor suppressor genes that are somatically mutated or deleted (except for *P27* and *P21*) (Adapted from[164]).

### Ras Pathway

Activation and deactivation of Ras, a guanosine-nucleotide–binding protein (G-protein), depends on the balance between active GTP-bound and inactive GDP-bound forms. Raf and PI3K are the downstream effectors of the active Ras-GTP protein, which culminates in survival, cell cycle progression, and migration[194]. Interestingly, mutations in *RAS* are infrequently found in GBM, nevertheless, increased Ras pathway activity is common in GBMs, which can be justified by upstream factors, such as RTK activation (EGFR or PDGFR)[195]. One of downstream effects of Ras is mitogen activated protein kinases (MAPK) signaling, which is also activated in GBM, contributing for cell proliferation[196]. Moreover, Ras signaling pathway can also be activated through the loss of *NF1*, found in 20% of GBMs (loss-of-function mutation).

### PI3K/PTEN/AKT Pathway

PI3K-mediated cell signaling pathway has been implicated in the pathogenesis of GBM. Activation of PI3K to the cell membrane leads to a downstream activation, such as AKT and mammalian target of rapamycin (mTOR), resulting in cell proliferation and increasing cell survival by apoptosis blocking[197]. In fact, this pathway appears to play a role in gliomagenesis, since 88% of GBM patients presented alterations in the EGFR/Ras/NF1/PTEN/PI3K pathway[198]. PI3K is negatively regulated by *PTEN*, a tumor-suppressor gene, which is frequently lost in GBM because of loss of heterozygosity (LOH) or its mutations (15-40%), leading to a constitutive activation of the PI3K pathway and higher levels of activated AKT in glioma cells[198].

### RB/CDKN2A-p16INK4a Pathway

RB/CDKN2A-p16INK4a pathway plays a pivotal role in the regulation of cell proliferation as well as cycle[199]. The RB1 protein controls the progression of the cell cycle through G1 into the S-phase. In proliferating cells, RB1 protein is phosphorylated by the CDK4⁄cyclin D1 complex which induces the release of E2F transcript factor that activates genes involved in the G1 to S-phase transition[200]. p16INK4a binds to CDK4, inhibits the CDK4⁄cyclin D1 complex, and consequently inhibits the G1 to S transition[200]. Thus, loss of normal RB1 function may result from altered expression of any of the *p16INK4a*, *CDK4*, or *RB1* genes.

In primary GBMs, the overall frequency of genetic alterations in the RB1 signaling pathway has been described to be 78% through *p16INK4a* homozygous deletion or mutations (52%), *p15INK4b* homozygous deletion (47%), *CDK4* amplification (18%), *RB1* mutation or homozygous deletion (11%), *p18INK4c* homozygous deletion (2%), *CCND2* amplification (2%), *CDK6* amplification (1%) Alterations in the p16INK4a⁄CDK4⁄RB1 pathway were unusual in oligodendrogliomas (4%) but were frequent in WHO grade III oligodendrogliomas (65%)[201].

### P53/MDM2/MDM4/CDKN2A-p14ARF Pathway

The TP53 protein is mainly involved the regulation of DNA damage, cell death, cell cycle, and differentiation. The TP53 is activated in response to cellular stress conditions, and consequently induces cell death in case of higher damage, in order to prevent mutated or damaged DNA cells from dividing, or activates DNA repair mechanisms[202]. TP53 transcriptionally regulates the promoter of p21, which blocks cell cycle progression by binding and inhibiting the function of cyclin-D proteins[203] TP53 and the RB pathways interact with each other via p21. It has also been demonstrated that TP53 regulates stem cells survival, proliferation, and differentiation, highlighting the relevance of TP53 in suppressing GBM[204]. After stress, TP53 activity is blocked by its negative regulator MDM2. However, inactivation of MDM2 by CDKN2A-p14ARF binding leads to activation of TP53. MDM4 (also called MDMX) also regulates TP53 activity, and p14ARF is negatively regulated by TP53.

In GBM, TP53 signaling pathway is disrupted as result of *TP53* mutation and/or amplification, and/or loss of expression of *CDKN2A-p14ARF*, and overexpression of *MDM2*, leading to uncontrolled cell proliferation and tumor formation[138, 205].

### Transforming growth factor-beta (TGF-β) signaling

TGF-β is a multifunctional cytokine that regulates cell growth, proliferation, differentiation, migration, extracellular matrix production and tissue homeostasis[206, 207]. The TGF-β superfamily includes various TGF-βs (TGF-β1, -β2, and -β3, which are highly homologous), bone morphogenetic proteins (BMPs), Nodal, Activin, growth and differentiation factors (GDFs), and anti-mullerian hormone (AMH) [208]. In mammals, there are three isoforms of TGF-β (TGF-β-1, -2 and -3). TGF-β first binds to TGF-β receptor II (TGF-βRII) altering its conformation. Then, TGF-βRII phosphorylates TGF-βRI, which in turn phosphorylates receptor-regulated (R-)Smad proteins (Smad 2, 3). Activated R-Smads form heteromeric complexes with Co-Smad and Smad-4, and are translocated to the nucleus (Figure 1.5[209]).

In tumorigenesis, TGF-β pathway has a dual role acting either as a tumor suppressor or a tumor promoter. TGF-β can promote proliferation, angiogenesis, invasion, metastasis and immune suppression[210]. TGF-β pathway plays a very crucial role in regulating the behavior of gliomas[211]. In fact, elevated levels of TGF-β have been reported in the blood serum of glioma patients and a remarkable correlation was observed between elevated TGF-β levels and high tumor grade, and poor patient outcome[212-215]. Moreover, this signaling pathway has been associated with glioma invasion, angiogenesis and immunosuppression[216-220]. Interestingly, TGF- β signaling was demonstrated to play a critical role in the maintenance of stemness in GBM stem cells (GSCs)[221-223].

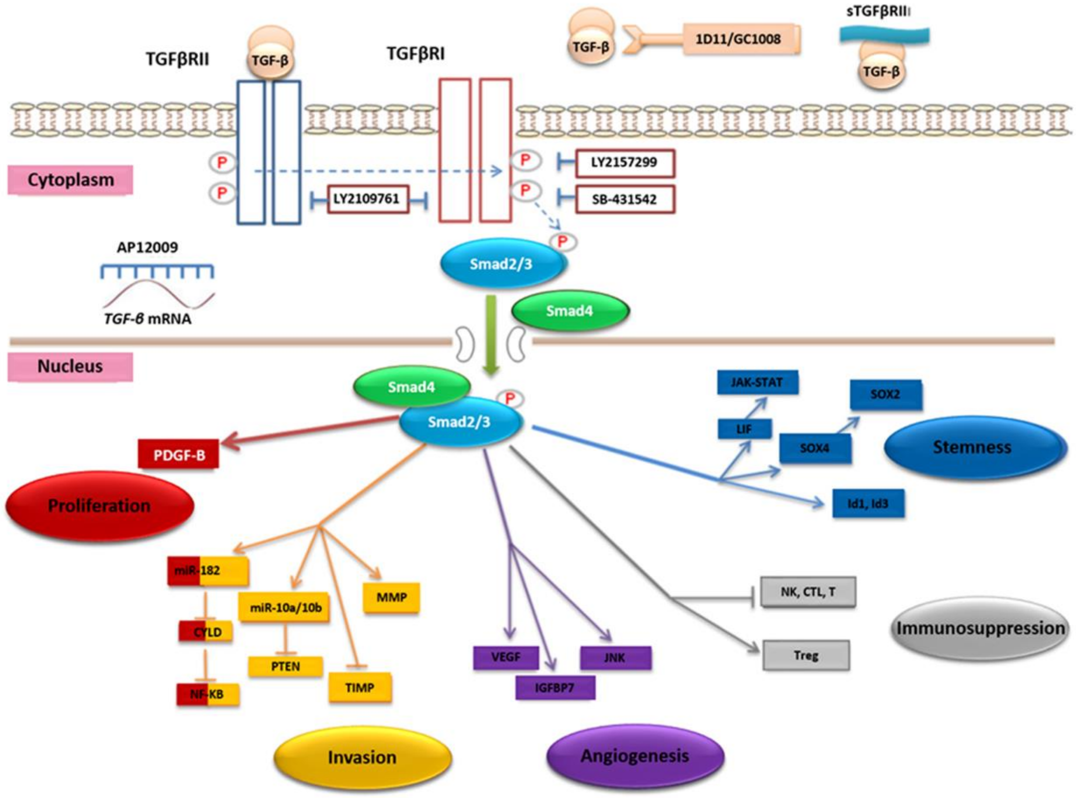

**Figure 1.5. TGF-β signaling in gliomagenesis**. The TGF-β signaling pathway contributes to glioma development through induction of multiple carcinogenic processes. This pathway promotes glioma proliferation via PDGF-B and miR-182, invasiveness via miR-182, miR-10 and MMP, as well as angiogenesis via VEGF, IGFBP7, and JNK. The TGF-β/Smads signaling pathway induces immunosuppression by inhibiting natural killer (NK) cells, cytotoxic T lymphocytes (CTL), dendritic cells (DC), and by upregulating T regulatory (Treg) cells. The TGF-β/Smads signaling pathway also drives GSC stemness via LIF, Sox4-Sox2, and Id1-Id3 (Adapted from[204]).

## 1.4 Glioma Stem Cells

CSCs or tumor initiating cells (TICs) are a subset of cells responsible for tumor initiation, progression and recurrence, and partly accountable for their marked heterogeneity, resistance to current therapies [224]. These cells share important characteristics with normal stem cells, including self-renewal, proliferation, and multipotency (i.e., capacity to differentiate into other cell lineages, such as astrocytes, oligodendrocytes and neurons). Therefore, eradication of CSCs is necessary to interrupt tumor expansion or prevent re-growth after therapy[179]. The first evidence of the existence of CSCs was reported for acute myeloid leukemia[225]. This tumor contained rare cells that when injected into immunodeficient mice recapitulated the hierarchy of differentiated leukemic cells[225].

In 2000, Uchida and co-workers isolated human NSPCs using CD133[226], which encourage the search for brain tumor stem cells (BTSCs). BTSCs were initially isolated from primary tumors by cell sorting based on CD133 expression[227]. Functionally, these CD133$^+$ tumor cells generated non-adherent neurospheres, had self-renewal capacity, a high proliferation potential, and were multipotent[227]. Additionally, CD133$^+$ BTSCs displayed a remarkable *in vivo* tumorigenicity when implanted in immunodeficient mice. As few as 100 CD133$^+$ tumor cells were able to originate tumors that recapitulated the parental tumor, whereas 100,000 CD133$^-$ BTSCs did not have that property[88]. These results provided robust evidence for a key role of CSCs in brain tumor biology. Several subsequent studies implicated them in resistance to RT and chemotherapy[228, 229]. The resilience of the tumors to recur after treatment has been linked to specific characteristics of CSCs, such as quiescent phenotype, enhanced DNA repair capacity, preferential activation of DNA damage checkpoint responses[228], and increased expression of drug efflux pumps and anti-apoptotic proteins[230]. This suggests that to achieve a complete durable response, the CSCs subpopulation must be specifically targeted in combination with currently available therapies.

Great efforts have been implemented towards the discovery, validation and use of CSCs enrichment methods. However, the heterogeneity of the tumor specimens, the rarity of the CSCs population, the expression of surface epitopes common to both non-CSCs and other cell types are limitations to the methods of isolation and propagation of CSCs[231].

### 1.4.1 Methods of Isolation/Identification of Glioma Stem Cells

GSCs are routinely isolated by cell sorting with specific cell-surface stem cell markers, as well as by *in vitro* enrichment using serum-free culture conditions supplemented with specific growth factors that allow neurospheres formation.

There are two methods of growing GSCs as an adherent monolayer or as nonadherent neurospheres cultures[232]. The most widely used method is the neurosphere-forming assay, similar to those used for culture of NSCs where cells are cultured in serum-free stem cell media supplemented with L-glutamine, B27, N2 and growth factors b-FGF (basic fibroblast growth factor) and EGF[233] (Figure 1.6). The neurospheres produced are heterogeneous aggregates derived from a single GSC or early progenitor cell with self-renewing and multipotency properties. Neurospheres derived from primary tumors express neural precursor markers, such as Nestin, CD133, Sox2, Musashi-1 and Bim [227, 234]. Despite the extensive use of the neurospheres-forming assay, this method presents some disadvantages. One of them is related to the low efficacy (1 to 30%) to establish GSC lines from primary tumors. This low efficacy results from the tendency of spheroid cells to spontaneously undergo differentiation and/or apoptosis during serial passages[235, 236]. Another limitation is that only a small percentage of cells within a neurosphere are true GSCs, while most cells are partially or fully differentiated progeny[236]. Additionally, it was shown that the selection of GSCs based on neurospheres culture fails to recapitulate the heterogeneity of the original tumor *in vivo* as assessed by gene expression, differentiation capacity and histological morphology[237-239], characteristics that GSCs maintained when isolated by markers expression[88]. In the second method, GSCs are grown as monolayers of adherent cells in laminin-coated cell culture plates in serum-free media supplemented with growth factors (Figure 1.6). These cells can be cultured for at least 1 year (>20 passages) without losing the stem cell properties and tumor initiation capacity[235]. The cells in this culture express NSC markers, such as Nestin, Sox2, and Olig2. These cells have the ability to differentiate into various lineages, including neuronal and glial, and are highly tumorigenic when implanted into the brains of immunodeficient mice[235]. There are a high percentage of true GSCs in the culture with significantly fewer differentiated or apoptotic cells. A possible explanation for this optimized result is the fact that all cells have equal access to the components of the media, a phenomenon that does not occur in tridimensional neurospheres cultures in which the center of the neurosphere may go necrotic.

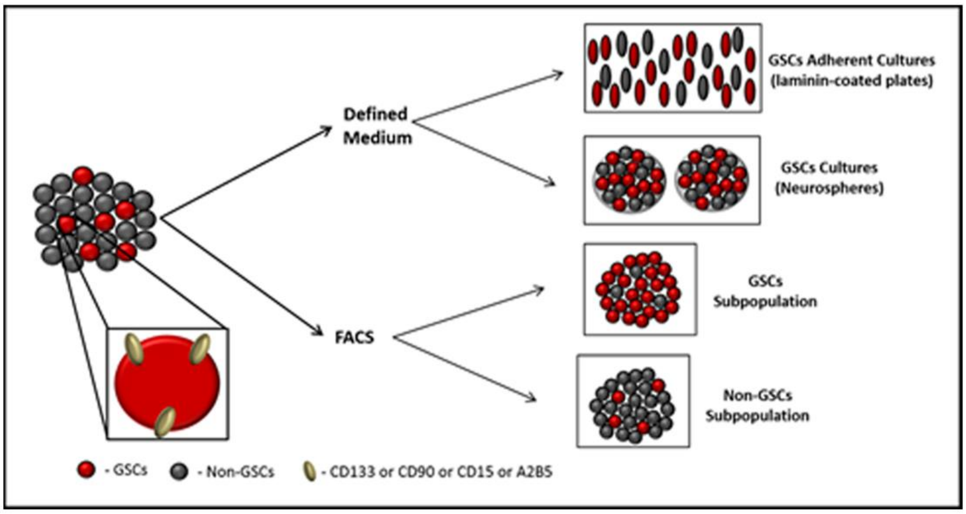

**Figure 1.6: Methods utilized for glioma stem cells (GSCs) isolation and culture.** GSCs (red cells) present in the tumor specimen are isolated either by fluorescence activated cell sorting (FACS) or MACS (magnetic activated cell sorting) using cell surface markers, such as CD133, CD15, CD90 and A2B5. Alternatively, isolated cells are directly cultured in a serum-free medium, typically supplemented with L-glutamine, B27, N2, and growth factors, that enriches for cells with stem cell properties.

Most GSCs markers have been appropriated from normal NSPC, such as, Bmi1, Musashi[240], Nanog[241, 242], Nestin[243], Sox2[240], among others[244, 245]. However, the use of intracellular proteins for GSCs enrichment by fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) has limitations. Therefore, several potential cell surface markers have been suggested, including CD133, CD15, A2B5, CD90, L1CAM and the combination of CD44 and ID1. FACS or MACS are the methods used for GSCs isolation and purification. These methods allow a purity at separation of 79.3-96.7% and 46.9-79.8%, respectively[227, 246].

CD133 (Prominin-1) is a 120-kDa five-transmembrane cell-surface protein expressed in NSCs, adult ependymal cells, and endothelial precursor cells[247]. This membrane bound glycoprotein is speculated to have a function in cell differentiation and EMT (epithelial to mesenchymal transition), however its confirmed function is still unknown[248, 249]. It is a CSC marker in a variety of tumors[250], including brain[88, 234]. The number of CD133$^+$ cells quantified by flow cytometry from human glioma samples, glioma sphere cultures, and established glioma cell lines is variable from low or rare[251, 252] to as high as 20% to 60%[246, 252, 253]. This variation may be explained by the recognition of inconstant glycosylated epitopes by the current available antibodies (AC133 or AC141)[254]. The expression of CD133 on cell surface marks GSCs and decreases with differentiation, however, the expression of *Prominin-1* mRNA is not regulated with stemness[255], suggesting that only the glycosylated surface protein CD133 is GSCs-dependent[256].

The expression of CD133 on the cell surface does not seem to be a requirement for neurospheres formation. Indeed, CD133$^-$ cells isolated from glioma specimens can have stem cell-like characteristics and tumorigenic potentia [233, 246]. Moreover, some CD133$^+$ cells lack GSC features, and are expressed by other cell types, including normal endothelial cells and endothelial glioma cells[231].

CD15, expressed in embryonic and adult neural stem/progenitor cells, is a putative useful marker for GSCs. It is also known as SSEA-1 (stage-specific embryonic antigen-1) or LeX (Lewis-X Antigen), a carbohydrate antigen associated with glycolipids and glycoproteins[246, 257]. CD15$^+$ cells are capable of self-renewal and multilineage differentiation, and have increased expression of the stem cells markers Bmi1 and Sox2[246]. In a mouse model of medulloblastoma[258], a subpopulation of cells that express CD15 can propagate tumors[259]. Moreover, CD15$^+$ cells isolated from GBMs are also highly tumorigenic, while CD15$^-$ cells present limited tumor formation capacity[260].

A2B5 is a cell surface ganglioside that is expressed by NSCs isolated from the subventricular zone of human embryos[261], and by NPCs from the subcortical white matter in the adult human brain[262]. In GBM and anaplastic astrocytoma, 33-90% of the cells express A2B5[263]. Two different studies demonstrated that A2B5$^+$ cells were able to form tumors in immunocompromised mice, while A2B5$^-$ cells were not able to do so[261, 263]. In addition, A2B5$^+$/CD133$^+$ and A2B5$^+$/CD133$^-$ subpopulations from glioma were capable of forming neurospheres *in vitro* and initiating tumors *in vivo*, suggesting that A2B5 is a GSC marker[261]. A cohort of genes and pathways significantly dysregulated in A2B5$^+$ tumor progenitor cells (TPCs), including *SIX1*, *EYA1* and *DACH2*, was identified by using A2B5 to isolate glioma TPCs, followed by messenger RNA profiling and comparison to A2B5$^+$ from normal white matter[264]. This set of genes is mostly expressed during development and not during adult life, what makes it particularly attractive for selective therapeutic target.

Another potential marker for GSCs is CD90 (Thy-1), a N-glycosylated glycophosphatidylinositol (GPI)-anchored cell surface protein, a known marker for bone marrow-derived and hematopoietic stem cells[265]. Recently, it was identified as a marker for human GSCs[266]. In GBM, 100% of the CD133$^+$ cells co-express CD90, but only a small portion of CD90$^+$ cells co-express CD133. Moreover, CD90 expression levels was significantly higher in high-grade than in low-grade gliomas[266].

Integrin-α6 is a member of the integrin family of extracellular matrix receptors for laminin and platelets. In the brain, this receptor regulates GSCs maintenance[267] and NSCs growth[268]. In GBM biopsies, cells positive for integrin-*α*6 were localized close to the tumor vasculature and co-expressed the stem cells markers CD133 and Nestin[269]. FACS sorting for integrin-*α*6 alone or in combination with CD133 led to an enrichment of cells with higher self-renewal capacity *in vitro*. Orthotropic injection of integrin-*α*6 positive cells in the brains of immunocompromised mice resulted in shorter survival when compared to integrin-*α*6 negative cells. Furthermore, shRNA-mediated knockdown of *integrin-α6* or treatment with integrin-blocking antibody reduced both neurospheres formation *in vitro* and tumor growth *in vivo*[269]. These findings strongly indicate a role for integrin-α6 in GSCs self-renewal and maintenance.

L1CAM (CD171) is a neural cell adhesion molecule that regulates neural cell growth, migration and survival during central nervous system development[270], however its role in normal adult nervous system is not clear. In gliomas, L1CAM is overexpressed and plays a role in tumor invasion[271, 272], being necessary for maintaining the survival and growth of CD133$^+$ cells with stem like properties[273]. Additionally, targeting *L1CAM* with lentiviral-mediated shRNA interference in CD133$^+$ glioma cells inhibited GSCs growth, neurospheres formation capacity and induced GSCs apoptosis. L1CAM knockdown decreased *olig2* expression and up-regulated the *p21* (*WAF1*/*CIP1*) tumor suppressor in $CD133^+$ glioma cells[273]. shRNA targeting of *L1CAM* expression *in vivo* suppressed tumor growth and increased animal's survival[273]. L1CAM-mediated signaling confers radiotherapy resistance in GSCs by improving Mre11, Rad50, and Nbs1 (MRN) complex function via Myc-NBS1-ATM axis and by leading to DNA checkpoint activation and DNA repair[274]. Therefore, L1CAM is a very promising GSCs marker and therapeutic target for GBM.

CD44, a multifunctional class I transmembrane glycoprotein, acts as a specific receptor for hyaluronic acid, promoting migration in normal cells and is highly expressed in several cancer types[275]. This cell surface marker is used to identify cancer stem in different tumor types, such as, breast cancer, pancreas, and prostate carcinomas[276-278]. Regarding GBM, Anido and colleagues[244] demonstrated that $CD44^{high}/Id1^{high}$ cells were located in perivascular niches of the tumor and possessed stem cell characteristics. They also showed that TGF-β pathway inhibition decreased the $CD44^{high}/Id1^{high}$ population through the repression of Id1 and Id3 levels, and prevented tumor initiation[244]. Additionally, high expression of both CD44 and ID1 conferred poor prognosis to GBM patients and were inversely correlated[244]. These results demonstrated that both CD44 and Id1 can be used to identify GSCs.

However, presumably no marker will be sufficiently robust to identify GSCs since different tissues contain multiple populations of stem cells that express different markers, therefore the use of a panel of molecular markers in combination with the discovery of novel specific antigens on the surface of GSCs will improve the purity, specificity of this cell population, and resolve controversies of the current *in vitro* and *in vivo* studies.

Besides the use of cell surface markers to isolate cancer stem cells from glioma tumors, the side population (SP) assay has also been used to identify and isolate GSCs. The SP is a subset of cells with differential efflux activity compared to the main cell population. This approach is based on the capacity that stem cells have of exporting the DNA binding Hoechst 33342 dye, while other cells take up the dye. This is due to the high expression levels of ATP-binding cassette (ABC) transporters MDR1 (ABCB1) and BCRP (ABCG2) in stem cells[279, 280] that bind ATP, hydrolyze it, and use the energy to transport several molecules across the plasma membrane. To identify the SP, cancer cells are stained with Hoechst 33342 dye, analyzed by flow cytometry and physically separated from the non-SP by FACS. Two emission wavelengths (red 675 nm and blue 450 nm) are used, and the small and non-stained cell population corresponds to the side population. The SP cells express high levels of stemness-related genes, and are able to generate multiple lineages[230]. However, a common limitation of this method is contamination by non-GSCs[281].

Although CSCs markers are widely used to identify and isolate GSCs, their expression is not sufficient to define this subpopulation. Therefore, is crucial to perform functional validations to confirm that the isolated cells really display the functional characteristics of GSCs (Figure 1.7[256]). Both *in vitro* and *in vivo* methods are used to assess these functional characteristics, such as sustained self-renewal, persistent proliferation, differentiation ability, as well as *in vivo* tumor initiation. It is important to have into consideration that, however, neurospheres forming assay evaluates both self-renewal and proliferation, this assay does not allow to test cellular hierarchy and does not replicate the tumor microenvironment. Therefore, in order to determine the capacity to recapitulate the original patient tumor and to evaluate the influence of microenvironment it is necessary to perform orthotropic transplantation of GSCs, being this *in vivo* assay the gold standard experiment for GSCs determination.

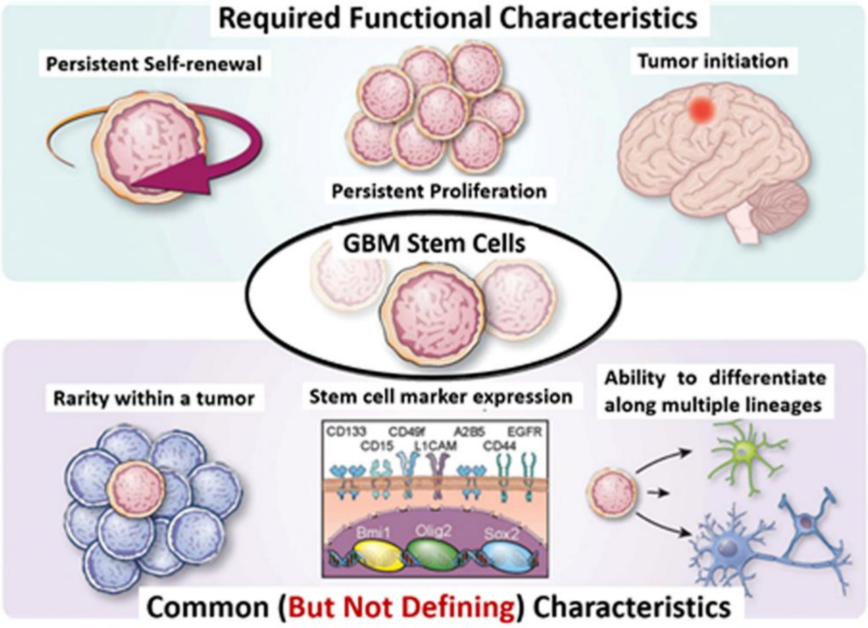

**Figure 1.7: Functional characteristics of GSC.** GSCs are defined by functional characteristics that include persistent self-renewal and proliferation, as well as tumor initiation upon secondary transplantation. GSCs also present additional characteristics such as stem cell marker expression (examples relevant to GBM and the brain are depicted), the ability to differentiate into multiple lineages, and the rarity within a tumor (Adapted from [256]).

### 1.4.2 Heterogeneity of Glioma Stem Cells

As observed for GBM, GSCs also have heterogeneity[282]. This heterogeneity is influenced by the localization of the tumor and the microenviromental clues within the tumor[283-

[285]. GSCs showed a mixture of cellular morphology when cultured as neurospheres[286, 287]. Moreover, diverse types of GSC were shown to be able to convert into each other within one GBM[282, 288]. The inter-tumoral heterogeneity of GSCs may contribute to the molecular classification of GBM. On a transcriptional level, Phillips *et al*. described proneural, proliferative, and mesenchymal GBM that corresponded to different stages of neurogenesis[92]. Particularly, proneural GBM included patients with younger age, primary diagnosis, and better prognosis. In contrast, more aged patients and patients with tumor relapses showed mesenchymal GBM[92, 289]. Later on, Verhaak, and colleagues, based on TCGA data, reproduced these subgroups and add two new subtypes, neural and classical[93]. Recent studies proposed similarities of CD133$^-$ CSC with the mesenchymal subtype and CD133$^+$ CSCs with the proneural subtype[252, 290]. Additionally, using a larger panel of CSC lines, results were corroborated, since CD133$^+$ GBM CSC lines corresponded to proneural GBM whereas CD133$^-$ GBM CSC lines corresponded to mesenchymal GBM. This suggests that the heterogeneity of GSCs corresponds to the heterogeneity of GBM[290]. More recently, Suva and co-workers identified a set of four transcription factors (POU3F2, Sox2, SALL2, and Olig2) in proneural GBM subtype that were able to reprogram differentiated tumor cells into GSCs. These transcription factors were required to maintain the tumor-forming capacity of these cells, suggesting that mediators of stem cell programs could capture the oncogenic capacity of GSCs[242]. By single-cell RNA sequencing, Patel and colleagues demonstrated that cells from the same tumor have differential expression of genes involved in oncogenic signaling, proliferation, hypoxia and immune response. They also identified novel genes predominantly present in GSCs when compared to differentiated cells from the same GBM tumor[90]. Moreover, an inverse correlation between stemness gradient and cell cycle signature was showed, indicating that stem cells as well as cells that grow in neurospheres divide more slowly compared with differentiated tumor cells[90]. Another study by single-cell functional analysis of patient GBM samples showed that individual clones presented unique proliferation and differentiation abilities, as well as a strong variation of genomics and response to therapy[89]. This work suggested that using functional clonal profile to identify drug-resistant tumor clones will lead to the discovery of new treatments.

## 1.5 Treatment of Malignant Glioma

### 1.5.1 Clinical Approaches on Glioma Treatment

Currently, the standard of care for low grade glioma includes RT as well as chemotherapy, however the timing of these treatments is still under investigation. Nevertheless, it is important to take into consideration that surgical resection continues to be the best option in the first phase of treatment, being associated with good outcomes and prognosis. Historically, this type of gliomas have been treated with external beam radiation, so far the gold-standard treatment for low-grade gliomas. Regarding chemotherapy, different regiments are being used such as, PCV (procarbazine, CCNU and vincristine), TMZ, and lomustine (CCNU), however these treatments remain controversial[12, 152]. Some studies revealed that the addition of adjuvant PCV chemotherapy was associated with a PFS benefit, but not with an OS benefit[291], while others showed that patients treated with both radiation plus PCV presented a median OS from 7.8 to 13.3 years[292]. Additional studies must include histologic type of tumor and molecular markers such as *1p19q* co-deletion and *IDH* mutations to assess treatment effect and target chemotherapeutics[293].

Regarding high grade gliomas treatment, a maximal possible and safe resection (preservation of neurologic function) continues to be the first step of treatment. In fact, it was demonstrated, in GBM patients, that surgical resection increases the OS in 4.2 months[294]. Currently, the standard of care includes the use of TMZ, an oral cytotoxic DNA-alkylating chemotherapy, with concomitant radiation therapy followed by adjuvant TMZ for 6 months[295]. This regime has been shown to improve in 2.5 months the median OS when compared to radiation alone (14.6 months compared to 12.1 months), with a two-fold increase in 2-year survival from 10.4 to 26.1%[295]. At recurrence, another treatment options consist in surgical resection with or without the placement of BCNU wafers, re-irradiation and chemotherapeutics such as nitrosoureas (CCNU, BCNU) or bevacizumab, a monoclonal antibody anti-VEGF. However, it is important to take into consideration that many limitations to the current chemotherapeutics in the treatment of glioma still remain: i) systemically delivered medications typically do not reach high concentrations within the CNS and at the site of the tumor, and ii) this type of treatments lead to significant systemic side effects such as myelosuppression[293].

### 1.5.2 Stem Cells-based Therapies for Glioma

The ultimate goal of cancer therapy is to target tumor cells without affecting normal cells. Currently, the conventional anti-tumor therapies used in treatment of glioma are essentially palliative and lack sensitivity. Therefore, there has been an urgent need to develop both new therapies and innovative ways to deliver those therapies.

Over the past decade, stem cells have been investigated has a relative new and promising therapeutic approach in the treatment of tumors, including gliomas. Stem cell-based therapies are considered attractive tools in cancer treatment because these cells present an intrinsic capacity to migrate towards sites of injury, including gliomas, are able to cross the blood brain barrier (BBB), can be genetically modified, and have immunosuppressive properties that may abrogate host immunoreaction following implantation [296-300];. Indeed, this selective cancer-tropism has been demonstrated for several stem cell types, including embryonic (ESCs), mesenchymal (MSCs), NSCs, endothelial, and hematopoietic[301].

It is accepted that the mechanism underlying the homing of stem cells to gliomas is similar to the mechanism underlying the homing of these cells to sites of injury. Injured tissues secrete factors, including chemokines, cytokines and growth factors that recruit stem cells by interact with membrane-bound receptors present in these cells. Therefore, it has been demonstrated, in a tumor context, including in glioma, that tumor cells are able to secrete chemokines, cytokines and growth factors that mediate the tumor tropism presented by stem cells. In an *in vitro* study, it has been verified that EGF, PDGFB, and stromal cell-derived factor 1α (SDF-1α), increased migration of MSCs towards glioma [302]. Additionally, other studies showed that TGF-β1, neurotrophin-3 (NT-3), interleukin (IL)-8, PDGF-D, TNF-α, VEGF-A also mediate glioma-tropic migration of MSCs[303-306]. Regarding NSCs, it has been revealed that monocyte chemotactic protein-1 (MCP-1), VEGF, VEGFR2, C-X-C chemokine receptor type 4 (CXCR4), and urokinase plasminogen activator receptor (uPAR) enhanced their migration towards gliomas[307-309]. Importantly, some studies demonstrated that therapeutic irradiation additional enhanced the tropism of MSCs towards glioma, via the inflammatory response[310, 311].

In order to generate stem cells presenting anti-tumor abilities, these cells can be modified in numerous ways: i) stem cells can be engineered to secrete therapeutic proteins that will act directly on tumor cells or indirectly on cells of the tumor microenvironment (Figure 1.8a); ii) stem cells can be modified to express a suicide gene which encodes an enzyme that converts a prodrug into a cytotoxic, inducing suicide of the SC and the death of tumor cells by the bystander effect (the movement of cytotoxin from the SC to adjacent cancer cells via a paracrine mechanism or gap junctions) (Figure 1.8b); iii) stem cells can be loaded with nanoparticles containing chemotherapeutic agents that are released in the tumor leading to cancer cells death (Figure 1.8c); and iv) stem cells can be infected with oncolytic viruses (OVs) that will replicate within the SCs, which will rupture and release the OV progeny that can infect tumor cells (Figure 1.8d)[301].

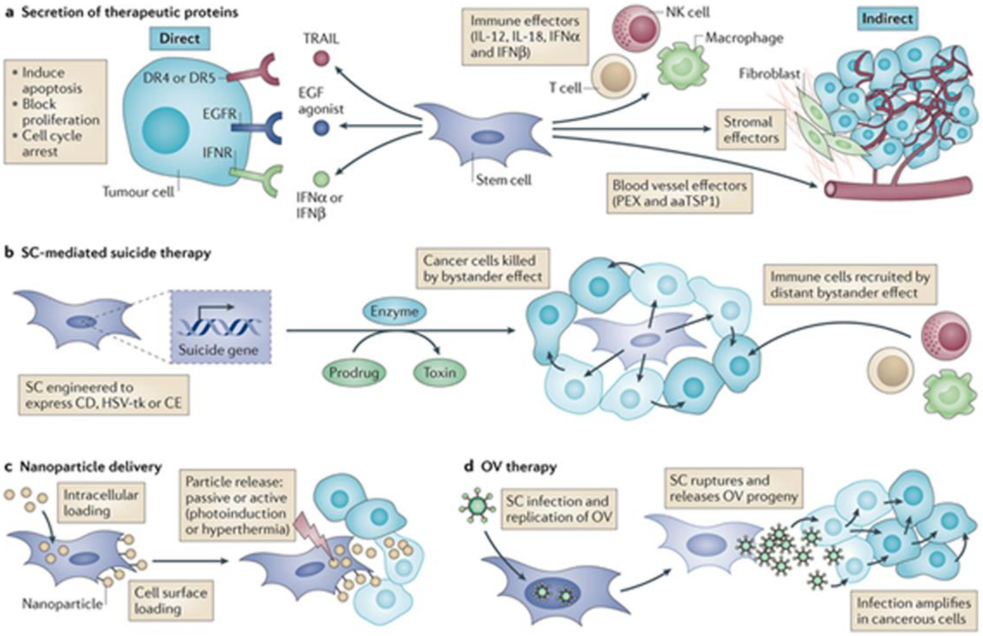

**Figure 1.8**: **Modifications of stem cells in order to promote tumor cell dead**. **a**) Stem cells can be modified in order to deliver therapeutic proteins For example, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), epidermal growth factor (EGF) agonists or interferons (IFNα or IFNβ) can be secreted  by stem cells to directly act on tumor cells presenting death receptor 4 (DR4) and DR5, EGF receptor (EGFR) or IFN receptors (IFNRs), respectively. On the other hand, stem cells can secrete immune, stromal or blood vessel effectors stromal; **b**) Stem cells can be engineered to mediate suicide gene therapy by expressing a suicide gene such as cytosine carboxylesterase (CE), deaminase (CD), or herpes simplex virus thymidine kinase (HSV-tk) which will convert a prodrug into a cytotoxic agent; **c**) Nanoparticles containing chemotherapy or imaging agents can be internalized into stem cells; **d**) Stem cells can be infected with oncolytic viruses (OVs) that can infect tumor cells and amplify infection. **Abbreviations:** TSP1, anti-angiogenic thrombospondin 1; IL, interleukin; NK, natural killer; PEX, a fragment of matrix metalloproteinase 2[301].

NSCs were the first stem cell type to be explored as stem cell-based therapies to deliver therapeutic agents to gliomas[300]. In this first study, the authors demonstrated that NSCs (genetically immortalized) had the capacity to migrate towards the main tumor mass and invading tumor cells that extended out of the tumor bulk[300]. Additionally, they demonstrated that these NSCs could be genetically modified to transport the therapeutic transgene for cytosine deaminase (CD; an enzyme that converts 5-fluorocytosine into 5-fluorouracil). Since this publication, numerous other works have used NSCs to deliver several anti-glioma agents, including tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), IL-23, IL-4, IL-8, antiangiogenic protein thrombospondin, and oncolytic viruses[300, 312-321].

NSCs are found in the subependymal zone of the lateral ventricles and the dentate gyrus of the hippocampus, and can differentiate into astrocytes, neurons, and oligodendrocytes[322]. These types of stem cells can be isolated from the brains of fetus or even from the adult brain, however it is not easy to rapidly expand, modify and characterize these cells in preparation for implantation into glioma patients that present a short overall survival. Therefore, the use of immortalized NSC lines that are readily available should be performed. In fact, in 2010, a clinical pilot trial using genetically engineered immortalized NSCs was performed for patients with recurrent high-grade gliomas, where NSCs were implemented at the time of surgery (https://clinicaltrials.gov/; identifier, NCT01172964).

Another type of stem cells that has been widely investigated as a stem cell-based therapy in the treatment of glioma are MSCs. Although both NSCs and MSCs present similar tumor tropism, infiltrative potential across BBB and can be genetically modified[320], MSCs presented some advantages, such as i) can be easily isolated and subsequently expanded *in vitro*; ii) present an immune privileged nature; iii) can be isolated from patients, making autologous transplant possible and avoiding immune-mediated rejection; and vi) no ethical issues are associated with their use[323-325].

#### 1.5.2.1 Potential of Mesenchymal Stem Cells-based application in Glioma Treatment

MSCs were first described more than four decades ago[326]. Friedenstein and colleagues defined these cells as plastic-adherent fibroblast colony-forming units with clonogenic capacity[326]. Presently and according with the International Society for Cellular Therapy (ISCT) criteria, MSCs have been defined as tissue-culture plastic adherent multipotent cells, since they can differentiate towards the osteogenic, chondrogenic and adipogenic lineages, presenting simultaneously self-renewal capacity; while displaying the expression of surface markers (CD105, CD73, CD90), and the downregulation of hematopoietic cell surface markers (CD45, CD34, CD14 or CD11b, CD79a or CD19 and Human Leukocyte Antigen DR)[327]. They are multipotent stem cells that can be isolated from adipose tissue (ASCs), bone marrow (BMSCs), umbilical cord Wharton's jelly (bulk-WJ-MSCs; perivascular region-human umbilical cord perivascular cells, HUCPVCs), dental pulp, placenta, amniotic fluid, umbilical cord blood, liver, lung, spleen, and brain[314, 328-330].

The first report that described the use of MSCs in the treatment of glioma was performed by Nakamura and colleagues[331]. Here, the authors demonstrated that MSCs derived from the bone marrow of rats could migrate toward syngeneic rat brain tumors derived (9L glioma cell line) via the corpus callosum after intracranial injection of the MSCs into the contralateral hemisphere. Interestingly, they observed that MSCs injected alone were able to decrease tumor burden and improved the OS of the animals. Moreover, it was also shown that these MSCs were able to deliver the anti-tumor cargo IL-2. The second study that evaluated MSCs as stem cell-based therapy was done by Nakamizo *et al*, where they showed that human MSCs derived from bone marrow had the ability to home human GBM xenografts derived from LN229, U251 and U87 after injection into the carotid artery of immunocompromised mice[302]. Additionally, they also observed an increased OS of tumor-bearing mice after administration of MSCs engineered to deliver IFN-β[302] . Several subsequent studies were performed confirming the ability of MSCs to migrate towards gliomas[301, 303, 304, 332-335]. However, it is not clear if this tropism of MSCs is associated with tumor promotion or suppression functions[336, 337]. Akimoto and colleagues showed that umbilical cord blood-derived MSCs induced apoptosis in glioma cells; however, in the same study, adipose-derived MSCs enhanced the growth of GBM cells[302, 303, 336, 338]. In another study, co-culturing of adipose-derived MSCs with human glioma cells led to higher survival and proliferation of glioma cells[339], whereas in another work, bone marrow-derived MSCs co-cultured with human glioma cells inhibited tumor cell proliferation[338]. Nevertheless, the rationale behind the modification of MSCs to express or deliver anti-glioma therapeutic agents, may be a promising therapeutic approach.

As NSCs, also MSCs were explored as cargo delivery vehicles in the treatment of glioma (Table 1.6). MSCs were engineered to deliver IL-2, IL-7, IL-18, IL-23, IFN-β, a modified IL-12, and TRAIL leading to glioma cell death as well as to improved animal overall survival, *in vivo*[302, 313, 335, 340]. Additionally, MSCs were also modified to secrete prodrug enzymes (also known as suicide gene therapy) that will kill the surrounding tumor cells. In glioma, some prodrug enzymes have been investigated as cargoes in MSCs: the rabbit carboxylesterase enzyme (rCE), cytosine deaminase (CD) and the herpes simplex virus/thymidine kinase (HSV/tk) (van Dillen[341-344]. MSCs have also been used to deliver oncolytic viruses, including conditionally replicating adenovirus (CRAd), delta-24-RGD oncolytic adenovirus [345, 346], as well as to be loaded with nanoparticles[347]. MSCs can also be used as antibody delivery vehicles (antibody against the EGFRvIII mutant form of EGFR) resulting in tumor vascularization reduction and increased glioma-bearing survival[348].

**Table 1.6: MSCs as stem cell-based therapy in the treatment of brain tumors**

| Transgene/modification | MSC source | Route | Glioma (source) | Ref |
|---|---|---|---|---|
| **IL-2** | Rat | IC/IT | 9L (rat) | [331] |
| **IL-12** | Human | IT | GL26 (mouse) | [328] |
| **IFN-β** | Human | IT/ICR | U87 (human) | [302] |
| **sTRAIL** | Human | IT | Gli36 (human) | [335] |
| **CD** | Human | IC | U87 (human) | [349] |
|  | Rat | IT | C6 (rat) | [350] |
|  | Rat | IT | 9L (rat) | [351] |
| **HSV-tk andVPA** | Human | IT | U87 (human) | [352] |
| **rCE** | Human | IT | F98 (rat) | [353] |
| **Endostatin** | Human | IT | U87 (human) | [329] |
| **CRAd** | Human | ICR | U87 (human) | [346] |
| **scFv anti-EGFRvIII** | Human | IT | U87 (human) | [348] |
| **Silica nanorattle-DOX** | Human | IT | U87 (human) | [354] |

**Abbreviations:** CD, cytosine deaminase; CRAd, conditionally replicating adenovirus; DOX, doxorubicin; EGFRvIII, epidermal growth factor receptor variant III; HSV-tk, herpes simplex virus type 1 thymidine kinase; IC, intracerebral; IT, intratumoral; ICR, intracarotid; IFN, interferon; IL, interleukin; LNCs, lipid nanocapsules; rCE, rabbit carboxylesterase enzyme; Ref, reference; scFv, single-chain antibody fragment; sTRAIL, soluble variant of tumor necrosis factor-related apoptosis-inducing ligand; VPA, valproic acid.

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# Chapter 2: Impact of *TGF-β1* -509C/T and 869T/C polymorphisms on glioma risk and patient prognosis

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RESEARCH ARTICLE

# Impact of *TGF-β1* -509C/T and 869T/C polymorphisms on glioma risk and patient prognosis

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**Abstract** Transforming growth factor beta (TGF-β) plays an important role in carcinogenesis. Two polymorphisms in the *TGF-β1* gene (-509C/T and 869T/C) were described to influence susceptibility to gastric and breast cancers. The 869T/C polymorphism was also associated with overall survival in breast cancer patients. In the present study, we investigated the relevance of these *TGF-β1* polymorphism in glioma risk and prognosis. A case-control study that included 114 glioma patients and 138 cancer-free controls was performed. Single nucleotide polymorphisms (SNPs) were evaluated by polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP). Univariate and multivariate logistic regression analyses were used to calculate odds ratio (OR) and 95 % confidence intervals (95 % CI). The influence of *TGF-β1*-509C/T and 869T/C polymorphisms on glioma patient survival was evaluated by a Cox regression model adjusted for patients' age and sex and represented in Kaplan-Meier curves. Our results demonstrated that *TGF-β1* gene polymorphisms -509C/T and 869T/C are not significantly associated with glioma risk. Survival analyses showed that the homozygous -509TT genotype associates with longer overall survival of glioblastoma (GBM) patients when compared with patients carrying CC+CT genotypes (OR, 2.41; 95 % CI, 1.06–5.50; $p=0.036$). In addition, the homozygous 869CC genotype is associated with increased overall survival of GBM patients when compared with 869TT+TC genotypes (OR, 2.62; 95 % CI, 1.11–6.17; $p=0.027$). In conclusion, this study suggests that *TGF-β1*-509C/T and 869T/C polymorphisms are not significantly associated with risk for developing gliomas but may be relevant prognostic biomarkers in GBM patients.

**Keywords** Glioma · Glioblastoma · Transforming growth factor beta 1 · Single nucleotide polymorphisms · Risk · Prognosis

## Introduction

During the last decades, the incidence and mortality of brain tumors have increased in most developed countries, mainly in the older age groups, with a slightly higher incidence in men than in women [1]. Gliomas, the most common primary tumors of the central nervous system (CNS), account for almost 80 % of brain malignancies [2]. According to their histological characteristics, these tumors can be divided into four main subgroups: astrocytomas, oligodendrogliomas, oligoastrocytomas, and ependymomas (the less common). Glioma tumors can also be divided into four grades of

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malignancy according to the World Health Organization (WHO) classification, being glioblastoma (GBM) the most common and biologically aggressive glioma type (grade 4) [3, 4]. Despite the advances in the field of neuro-oncology, the prognosis of glioma patients remains very poor [5], particularly for patients with GBM [6]. Few factors have been associated with increased glioma risk, including hereditary syndromes, such as Li-Fraumeni and Turcot syndromes, neurofibromatosis (type 1 and type 2) and tuberous sclerosis complex [7, 8], familial aggregation [7, 9], and exposure to high doses of ionizing radiation [7, 10, 11]. Some genome-wide association studies have showed that single nucleotide polymorphisms (SNPs) are associated with glioma susceptibility [12, 13]. However, other factors that may contribute to glioma susceptibility require additional investigation.

The transforming growth factor beta (TGF-β), a multifunctional cytokine, is involved in the regulation of several immunomodulatory processes that play a key role in numerous cellular processes, such as proliferation, differentiation, apoptosis, angiogenesis, tumor progression, and extracellular matrix production [14]. TGF-β has three isoforms, TGF-β1, TGF-β2, and TGF-β3. These three isoforms bind and activate a membrane receptor serine/threonine complex (type I TGF-βRI and type II TGF-βRII). The intracellular signaling is initiated when TGF-βRII phosphorylates TGF-βRI, which in turn phosphorylates the transcription factors Smad2 or Smad3 that consequently bind Smad4. This complex is translocated from the cytoplasm to the nucleus, resulting in the transcriptional activation of TGF-β responsive genes that ultimately mediate the effects of TGF-β at the cellular level [15]. Deregulation of TGF-β signaling has been implicated in cancer, where TGF-β has been demonstrated to have a dual role. It may act as a strong inhibitor of proliferation of normal astrocytes and epithelial cells, being considered a tumor suppressor factor, but in some tumor types, including high-grade glioma, TGF-β acts as an oncogenic factor contributing to cell growth and invasion and decreases host immune responses against tumor [16]. It was also demonstrated that TGF-β activity confers poor prognosis in glioma patients [17, 18]. Several studies have identified *TGF-β1* as a predictive cancer biomarker, particularly focusing on *TGF-β1* genetic polymorphisms [19–22]. In fact, it was demonstrated that polymorphisms in this gene contribute to breast and gastric cancers susceptibility [19, 23]. Additionally, studies demonstrated an association of *TGF-β1* 869T/C polymorphism with overall survival of breast cancer patients [24, 25]. The *TGF-β1* gene is located on chromosome 19q13, and two common polymorphisms of the *TGF-β1* gene have been extensively studied, the -509C/T (rs1800469) and the 869T/C (rs1800470, previously known as rs1982073; T29C and Leu10Pro) [22, 26, 27]. The -509C/T polymorphism is located in the promoter region of *TGF-β1* gene, which may potentially regulate *TGF-β1* transcription. The 869T/C polymorphism is located in exon 1 and could lead to a leucine-to-proline substitution at codon 10 [21, 26]. Some studies demonstrated that the -509T allele is associated with an increased transcriptional activity as compared to -509C allele [28], which leads to a higher serum concentration of TGF-β1 among TT homozygotes than in the CT heterozygotes [29]. Similarly, the 869C allele was associated with high serum concentrations of TGF-β1 [19, 30]. Moreover, some studies showed that -509C/T and 869T/C *TGF-β1* polymorphisms were able to affect TGF-β1 protein expression [31, 32]. Importantly, the circulating levels of this cytokine have been associated with cancer [33–35]. The relevance of *TGF-β1* polymorphisms has not been reported in gliomas. Thus, the aim of this case-control study was to investigate the relevance of *TGF-β1* -509C/T and 869T/C polymorphisms in glioma susceptibility and how specific polymorphic variants may influence the prognosis of patients.

## Methods

### Study population

In this case-control study, we enrolled 114 glioma patients from Portugal (Hospital of Braga, Braga, and Hospital São João, Porto) diagnosed between 2004 and 2013. The peripheral blood from these subjects was collected. Tumors were classified according to WHO [3], and clinico-pathological features are summarized in Table 1. The control group was randomly selected from blood donors at Hospital of Braga, and it included 138 cancer-free individuals. All subjects were of Caucasian ethnic background. The procedures followed in the present study were in accordance with institutional ethical standards.

### Genotyping

Genomic DNA from glioma cases and controls was extracted from peripheral blood leukocytes by proteinase K/chloroform/isopropanol treatment [36]. The purified DNA was used to determine the genotypes of both polymorphisms, using polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) methods. The PCR primers for -509C/T polymorphism were 5′-CCCGGCTCCATTTCCAGGTG-3′ (forward) and 5′-GGTCACCAGAGAAAGAGGAC-3′ (reverse), and for the 869T/C polymorphism were 5′-CCTCCCCACCACACCAG-3′ (forward) and 5′-CCGCAGCTTGGACAGG-3′ (reverse). The PCR was performed in a total volume of 25 μl containing 50 ng of DNA, 0.5 U of KAPA Taq DNA polymerase (GRiSP), 1× KAPA Taq Buffer A containing $MgCl_2$, 0.2 mM dNTP mix, and 0.8 μM of each primer. For the -509C/T polymorphism, the DNA was initially

**Table 1** Clinico-pathological features of gliomas and controls

| Groups (WHO grade) | Number of cases | Age, year (mean±SD) | Male/female ratio |
|---|---|---|---|
| Controls | 138 | 40.9±12.1 | 1.2 |
| Gliomas (2–4) | 114 | 58.3±12.9 | 1.7 |
| Astrocytomas (2–4) | 97 | 58.9±12.7 | 2.2 |
| Astrocytomas (2–3) | 8 | 52.3±13.3 | 1 |
| Diffuse astrocytomas (2) | 5 | 54.8±13.8 | 0.67 |
| Anaplastic astrocytomas (3) | 2 | 55.0±9.9 | 1 |
| Gliosarcomas (4) | 4 | 61.3±9.1 | All males |
| Glioblastomas (4) | 85 | 59.5±12.7 | 2.1 |
| Oligodendrogliomas (2–3) | 16 | 53.1±12.3 | 0.45 |
| Oligodendrogliomas (2) | 4 | 46.3±9.7 | All females |
| Anaplastic Oligodendrogliomas (3) | 10 | 54.7±12.9 | 0.67 |

denatured at 95 °C for 7 min, followed by 11 cycles of 95 °C for 30 s, 66–61 °C for 30 s, and 72 °C for 1 min, followed by 30 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 1 min. The PCR was finished by a final extension cycle at 72 °C for 8 min. Regarding 869T/C polymorphism, the PCR cycle conditions consisted of an initial denaturation step at 95 °C for 5 min, followed by 9 cycles of 95 °C for 30 s, 68–64 °C for 30 s, and 72 °C for 30 s, followed by 30 cycles of 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 30 s. Finally, the PCR was completed by a final extension cycle at 72 °C for 8 min. After confirmation of an amplified fragment of the expected size (808 bp for -509C/T and 235 bp for 869T/C) on 2 % agarose gel, 8–12 μL of PCR products were digested overnight at 37 °C with the appropriate restriction enzymes. For the -509C/T polymorphism, 10 U of restriction enzyme Bsu36I (New England Biolabs) was used, and for the 869T/C, 5 U of the restriction enzyme MspA1I (Fermentas) was applied. The DNA fragments were resolved on 2 % agarose gel for -509C/T polymorphism and 4 % agarose gel for 869T/C polymorphism and were detected by Greensafe Premium staining (Nzytech). For -509C/T polymorphism, the PCR product (808 bp) with C allele was digested into two fragments (617 and 191 bp), whereas the PCR product with T allele was not digested by Bsu36I. For 869T/C polymorphism,

**Table 2** Univariate analysis of the association between -509C/T and 869T/C polymorphisms and risk for each glioma group

| Polymorphism | Control | Glioma (WHO grades 2–4) | OR (95 % CI)[a] | Glioblastoma (WHO grade 4) | OR (95 % CI)[a] |
|---|---|---|---|---|---|
| *TGF-β1*-509C/T | | | | | |
| Genotypes | | | | | |
| TT | 22 | 18 | – | 16 | – |
| CC | 54 | 42 | 0.95 (0.45–1.98) | 28 | 0.71 (0.32–1.57) |
| CT | 62 | 54 | 1.07 (0.52–2.19) | 41 | 0.91 (0.43–1.94) |
| CC+CT | 116 | 96 | 1.01 (0.51–2.00) | 69 | 0.82 (0.40–1.66) |
| Alleles | | | | | |
| T | 0.384 | 0.395 | – | 0.429 | – |
| C | 0.616 | 0.605 | 0.97 (0.68–1.39) | 0.571 | 0.85 (0.57–1.25) |
| *TGF-β1* 869T/C | | | | | |
| Genotypes | | | | | |
| CC | 26 | 19 | – | 17 | – |
| TT | 48 | 42 | 1.20 (0.58–2.47) | 30 | 0.96 (0.45–2.05) |
| TC | 64 | 53 | 1.13 (0.57–2.27) | 38 | 0.91 (0.44–1.89) |
| TT+TC | 112 | 95 | 1.16 (0.61–2.23) | 68 | 0.93 (0.47–1.84) |
| Alleles | | | | | |
| C | 0.420 | 0.399 | – | 0.424 | – |
| T | 0.580 | 0.601 | 1.09 (0.76–1.56) | 0.576 | 1.10 (0.75–1.64) |

[a] Odds ratio (OR) with 95 % confidence intervals (CI)

the PCR product (235 bp) with T allele was digested into four fragments (103, 67, 40, and 25 bp), and the PCR product with C allele was digested into five fragments (91, 67, 40, 25, and 12 bp).

### Statistical analysis

Data analysis was performed using SPSS 22.0 software (SPSS, Inc.). Differences in allele and genotype frequencies were compared between glioma patients and cancer-free controls by the chi-square test, and the frequency distribution of age and sex was compared between glioma patients and cancer-free controls by the nonparametric Wilcoxon-Mann Whitney test. Additionally, the chi-square test was used to verify that the observed allele distribution, in the control group, was in Hardy-Weinberg equilibrium. Odds ratio (OR) and 95 % confidence intervals (95 % CI) were estimated by univariate and multivariate logistic regression analyses, adjusted for patients' age (as a continuous variable) and sex, to assess the risk for each glioma type conferred by a particular allele and genotype of each polymorphism. Patient survival curves were assessed by the Kaplan-Meier method for GBM. A Cox regression model adjusted for patients' age (as a continuous variable) and sex was applied to evaluate the effect of the *TGF-β1* genotypes on overall survival. Statistical significance was considered for *p* values <0.05.

## Results

The clinico-pathological features of the controls and cases are summarized in Table 1. For both *TGF-β1*-509C/T and 869T/C polymorphisms, 114 glioma patients and 138 cancer-free control individuals were analyzed. The statistical analysis of age distribution between control and glioma cases showed significant differences ($p \leq 0.001$). Regarding sex distribution, no significant differences were found between controls and cases ($p=0.195$). The genotype and allele frequencies of the *TGF-β1*-509C/T and 869T/C polymorphisms in controls and glioma cases are shown in Table 2. The frequencies of the CC,

**Table 3** Multivariate logistic regression analysis of the association between -509C/T and 869T/C polymorphisms and risk for each glioma group

| Polymorphism | Control | Glioma (WHO grade 2–4) | OR (95 % CI)[a] | Glioblastoma (WHO grade 4) | OR (95 % CI)[a] |
|---|---|---|---|---|---|
| *TGF-β1*-509C/T | | | | | |
| Genotypes | | | | | |
| TT | 22 | 17 | – | 15 | – |
| CC | 54 | 40 | 1.14 (0.45–2.98) | 27 | 0.76 (0.27–2.13) |
| CT | 62 | 52 | 1.11 (0.45–2.75) | 39 | 0.82 (0.30–2.21) |
| CC+CT | 116 | 92 | 1.13 (0.48–2.63) | 66 | 0.79 (0.31–2.01) |
| Alleles | | | | | |
| T | 0.384 | 0.394 | – | 0.426 | – |
| C | 0.616 | 0.606 | 1.08 (0.68–1.70) | 0.574 | 0.89 (0.53–1.48) |
| Age | | | **1.12 (1.09–1.15)** | | **1.14 (1.10–1.18)** |
| Sex | | | | | |
| Male | 76 | 69 | – | 58 | – |
| Female | 62 | 40 | **0.40 (0.20–0.77)** | 27 | **0.24 (0.11–0.52)** |
| *TGF-β1* 869T/C | | | | | |
| Genotypes | | | | | |
| CC | 26 | 17 | – | 15 | – |
| TT | 48 | 40 | 1.36 (0.55–3.34) | 29 | 0.89 (0.33–2.41) |
| TC | 64 | 52 | 1.12 (0.47–2.66) | 37 | 0.80 (0.31–2.07) |
| TT+TC | 112 | 92 | 1.22 (0.55–2.73) | 66 | 0.84 (0.35–2.02) |
| Alleles | | | | | |
| C | 0.420 | 0.394 | – | 0.414 | – |
| T | 0.580 | 0.606 | 1.19 (0.75–1.87) | 0.586 | 1.09 (0.64–1.84) |
| Age | | | **1.12 (1.09–1.15)** | | **1.14 (1.10–1.18)** |
| Sex | | | | | |
| Male | 76 | 69 | – | 58 | – |
| Female | 62 | 40 | **0.39 (0.20–0.76)** | 27 | **0.24 (0.11–0.52)** |

[a] Odds ratio (OR) with 95 % confidence intervals (CI), adjusted for age (as a continuous variable) and sex. Boldfaced values indicate significant differences at 5 % level

CT, and TT genotypes of -509C/T were 39.1, 44.9, and 16.0 % in cancer-free controls, and 36.8, 47.4, and 15.8 % in glioma patients, respectively. Regarding the 869T/C polymorphism, the frequencies of the TT, TC, and CC genotypes were 34.8, 46.4, and 18.8 % in controls, and 36.8, 46.5, and 16.7 % in glioma cases, respectively. The distribution of -509C/T and 869T/C allele frequencies in the control group were in Hardy-Weinberg equilibrium ($p$=0.891 and $p$=0.685, respectively).

When assessing the allele frequencies of the *TGF-β1*-509C/T polymorphism by univariate analysis, we found that the C allele was not significantly associated with a higher risk for glioma (OR, 0.97; 95 % CI, 0.68–1.39; Table 2). Additionally, using TT genotype as reference, the OR analysis showed that the CC, CT, and combined CC+CT genotypes were not significantly associated with increased risk for glioma (OR, 0.95; 95 % CI, 0.45–1.98 for CC; OR, 1.07; 95 % CI, 0.52–2.19 for CT; OR, 1.01; 95 % CI, 0.51–2.00 for CC+CT; Table 2). Evaluating the *TGF-β1* 869T/C polymorphism by univariate analysis, the T allele was not significantly associated with a higher risk for glioma (OR, 1.09; 95 % CI, 0.76–1.56; Table 2). Using CC genotype as reference, the OR analysis showed that the TT, TC, and combined TT+TC genotypes were not significantly associated with increased risk for glioma (OR, 1.20; 95 % CI, 0.58–2.47 for TT; OR, 1.13; 95 % CI, 0.57–2.27 for TC; OR, 1.16; 95 % CI, 0.61–2.23 for TT+TC; Table 2). Taking into account that GBM were the most frequent subtype in our series ($n$=85), we also compared the control group with GBM cases. Using similar analysis, a lack of association between both *TGF-β1*-509C/T and 869T/C allele or genotype variants and risk for developing GBM was observed (Table 2). Moreover, for both polymorphisms, a multivariate logistic regression model adjusted for sex and age as a continuous variable (Table 3) was applied. As expected, increased age was associated with increased risks for developing glioma and GBM. Similarly, female gender was associated with decreased risks (Table 3). Consistent with the results observed by the univariate analysis, no associations between each polymorphic variant and risk for developing gliomas or GBMs were found (Table 3).

We then evaluated whether these *TGF-β1* polymorphisms may have an impact in patients' survival. To do so, we focused exclusively in GBM patients with available survival data ($n$=44), as glioma grade is a strong influencer of survival, precluding an analysis in the whole glioma dataset. Regarding -509C/T polymorphism, the Cox model showed that GBM patients carrying the TT genotype had significantly increased overall survival compared to those with the CC+CT genotypes (OR, 2.41; 95 % CI, 1.06–5.50; Table 4; $p$=0.036, Fig. 1a). Moreover, patients with CT genotype alone presented a shorter overall survival when compared to those carrying TT genotype (OR, 2.72; 95 % CI, 1.12–6.65; Table 4; $p$=0.028, Fig. 1b). No significant differences in overall survival were found in GBM patients with *TGF-β1*-509CC versus TT genotypes (Table 4). Concerning the survival analysis for the *TGF-β1* 869T/C polymorphism, the Cox regression model demonstrated that TT+TC genotypes were significantly associated with shorter survival in GBM patients, as compared to the CC genotype (OR, 2.62; 95 % CI, 1.11–6.17; Table 4; $p$=0.027, Fig. 1c). These results were further supported when we compared patients with TC genotype with patients carrying the CC genotype (OR, 2.71; 95 % CI, 1.12–6.54; Table 4; $p$=0.027, Fig. 1d). No significant differences in overall survival were found in GBM patients with *TGF-β1* 869CC versus TT genotypes (Table 4).

## Discussion

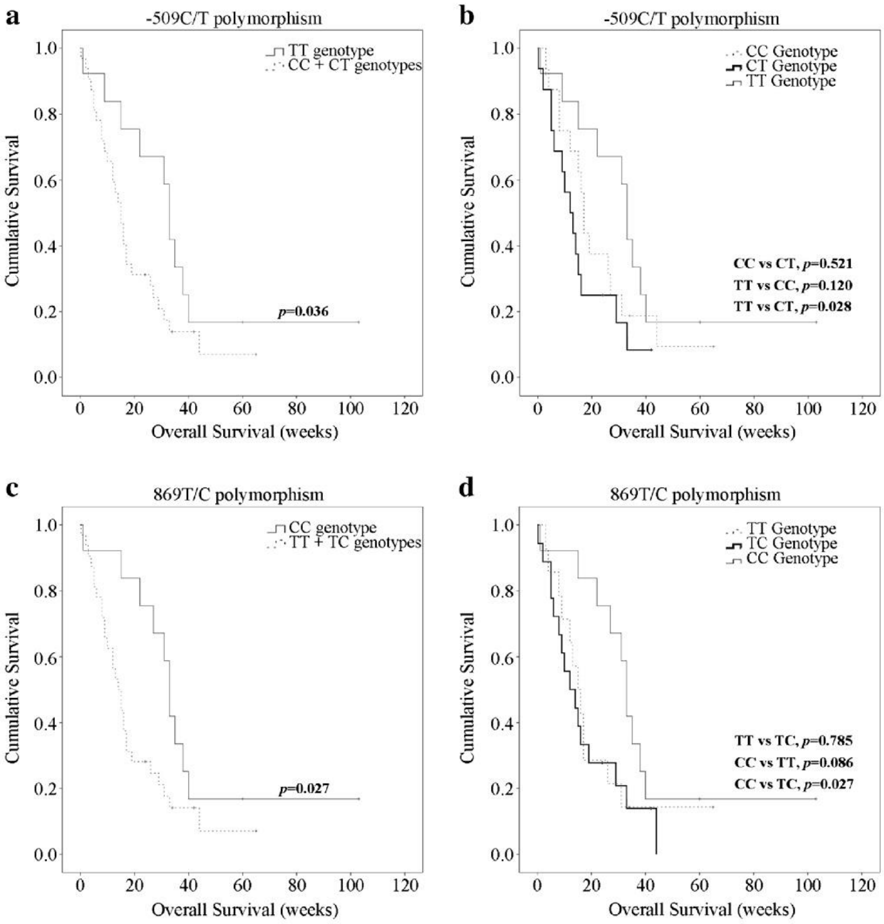
Gliomagenesis is a complex and poorly understood process in which genetic and environmental factors play critical roles. Several studies have suggested that SNPs are the most common sources of human genetic variation, and they may contribute to individual's susceptibility to cancer, including glioma [37]. So far, SNPs of several genes have been studied and identified as putative biomarkers for glioma susceptibility. Some examples include genes encoding proteins involved in

**Table 4** Multivariate COX regression analysis of the association between -509C/T and 869T/C polymorphisms and survival in grade 4 gliomas

| Polymorphism | Number of cases | OR (95 % CI)[a] |
|---|---|---|
| *TGF-β1*-509C/T | | |
| Genotypes | | |
| TT | 12 | – |
| CC | 16 | 2.09 (0.83–5.31) |
| CT | 16 | **2.72 (1.12–6.65)** |
| CC+CT | 32 | **2.41 (1.06–5.50)** |
| Age | | 1.02 (0.99–1.05) |
| Sex | | |
| Male | 31 | – |
| Female | 13 | 1.13 (0.52–2.45) |
| *TGF-β1* 869T/C | | |
| Genotypes | | |
| CC | 12 | – |
| TT | 14 | 2.43 (0.88–6.70) |
| TC | 18 | **2.71 (1.12–6.54)** |
| TT+TC | 32 | **2.62 (1.11–6.17)** |
| Age | | 1.01 (0.99–1.04) |
| Sex | | |
| Male | 31 | – |
| Female | 13 | 1.28 (0.57–2.89) |

[a] Odds ratio (OR) with 95 % confidence intervals (CI), adjusted for age (as a continuous variable) and sex. Bold-faced values indicate significant differences at 5 % level

**Fig. 1** Effect of *TGF-β1*-509C/T and 869T/C polymorphisms in the survival of glioblastoma patients. Kaplan-Meier overall survival curves for *TGF-β1* -509C/T (**a**, **b**) and 869T/C (**c**, **d**) polymorphisms. In the -509C/T polymorphism, Cox regression analysis showed that the group of glioblastoma patients harboring CC−CT genotypes (**a**, $p=0.036$) or patients with CT genotype (**b**, $p=0.028$) had statistically significant shorter overall survivals when compared to patients with TT genotype. Regarding the 869T/C polymorphism, the group of glioblastoma patients with TT+TC genotypes (**c**, $p=0.027$) or patients with TC genotype (**d**, $p=0.027$) had significantly shorter overall survivals than patients with CC genotype. *Tick marks* indicate censored data



DNA repair pathways (*MGMT, PRKDC, ERCC1, XRCC1, APEX1, TP53, PARP1*, and *LIG1*) [38–41], cancer metabolism (*GST, CYP2D6, SOD2, SOD3, GPX1*, and *NOS1*) [42, 43], growth pathways [44, 45], among others [46, 47]. Many association studies on the *TGF-β1* polymorphisms have been conducted in several types of cancer, including lung [27], prostate [20, 26], gastric [21], hepatocellular [22], and breast cancers [19, 24, 30, 48]. To the best of our knowledge, this is the first study to evaluate the *TGF-β1*-509C/T and 869T/C polymorphisms in glioma patients. This is particularly relevant as these two polymorphisms have been reported to affect TGF-β1 protein expression and influence the structure and function of TGF-β1 peptide which may contribute to cancer [31, 32].

Using both univariate and multivariate statistical analyses, our results showed that none of the *TGF-β1*-509C/T and 869T/C polymorphisms are significantly associated with glioma susceptibility. These data fit well with previous studies in other tumor types in which 869T/C was not associated with breast cancer risk [48], and -509C/T polymorphism was not associated with an increased risk of colorectal cancer [49]. While in our dataset, we included solely patients of Caucasian background, future studies should evaluate how these *TGF-β1* polymorphisms may have relevance in other ethnic backgrounds, as previously suggested for many other polymorphisms [50–53].

It has been described that TGF-β1 contributes to cell growth, angiogenesis, and invasion, is highly active, and confers poor prognosis in high-grade glioma patients [16–18]. Therefore, it is conceivable that patients carrying the T allele of the -509C/T polymorphism and patients with the C allele of the 869T/C polymorphism may have reduced cancer survival, since both these alleles are associated with an elevated *TGF-β1* levels. Contrarily, in our study, GBM patients carrying TT genotype of the -509C/T polymorphism and patients with CC genotype of 869T/C polymorphism presented longer overall survival. This is in agreement with a previous work where it has been shown that breast cancer patients carrying the CC genotype of the 869T/C polymorphism presented a longer overall survival [24]. Therefore, the TT genotype of the -509C/T polymorphism and the CC genotype of the 869T/C polymorphism have the potential to be used as predictive marker of better survival in patients with GBM. Additionally, taking into account that -509T allele has been suspected to increase the transcription of *TGF-β1*, patients that present this variant may be more suited for an anti-TGF-β1 monoclonal antibody therapy (Metelimumab) [54]. It remains, however, to be seen if -509C/T and 869T/C polymorphisms are in a linkage disequilibrium and if it is functionally relevant. For instance, it has been shown that these two *TGF-β1* polymorphisms (-509C/T and 869T/C) are in strong linkage disequilibrium in breast cancer patients, although it remains to be determined which of the two polymorphisms is functionally significant and affect survival [25].

In conclusion, this study shows that *TGF-β1*-509C/T and 869T/C polymorphisms do not confer susceptibility to develop glioma but may have an impact in the survival of GBM patients. Specifically, the *TGF-β1*-509TT and 869CC genotypes can be used as predictive markers of improved survival. In the future, additional studies with larger datasets will be needed to extend and validate these novel findings.

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**Conflict of interest** None.

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# Chapter 3: Intracellular autofluorescence as a new biomarker to identify Glioblastoma Stem Cells

The results presented throughout this chapter are in final phase of preparation for submitting a manuscript to an international peer reviewed journal:

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# Intracellular autofluorescence as a new biomarker to identify Glioblastoma Stem Cells

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## Conflict of Interest

The authors disclose no potential conflicts of interest.

## Abstract

Glioblastoma stem cells (GSCs) have paramount roles in tumor initiation, progression, recurrence, and therapy resistance. Thus, GSCs must be specifically targeted to achieve improved clinical responses of glioblastoma (GBM) patients.

Several markers have been proposed to identify/isolate GSCs, including CD133 and CD15. However, these markers lack full specificity and sensitivity (seem valid only for subsets of GBMs), and no generally accepted universal marker for GSCs has been defined yet, highlighting the need for the discovery of new markers. In the context of the recent identification of a subpopulation of cells with cancer stem cells (CSCs) features presenting an autofluorescent subcellular compartment in a variety of epithelial cancers, we proposed to evaluate whether this phenotype is also present in GBM, and identifies GSCs. Our work identifies a subpopulation of autofluorescent (Fluo$^+$) GBM cells, both in established and primary GBM cells. Functionally, Fluo$^+$ cells present typical features of GSCs, including higher capacity to grow as 3D neurospheres, long-term self-renewal ability, and increase expression of several stem cell and pluripotency-associated genes. In addition, exposure of GBM cells to temozolomide (TMZ) chemotherapy or to radiation treatment lead to a significant enrichment of the Fluo$^+$ cells' population in all tested models. Importantly, *in vivo* orthotopic models showed that mice with intracranial tumors derived from Fluo$^+$ GBM cells have a significantly shorter overall survival than those with non-autofluorescent (Fluo$^-$) GBM cells, further highlighting the GSC-associated malignant phenotype of Fluo$^+$ cells. Mechanistically, and similarly to CSCs from carcinomas, the autofluorescent phenotype of GSCs is due to the accumulation of riboflavin in cytoplasmic vesicles bearing ATP-dependent ABCG2 transporters. In conclusion, our work identified an intrinsic autofluorescent phenotype present in GBM cells with GSCs features, which can be straightforwardly used as a novel marker in these highly-malignant and therapy-insensitive tumors.

**Keywords:** Glioblastoma; Glioblastoma stem cells; Stem cell markers; Autofluorescence; Riboflavin; ABCG2

## 3.1 Introduction

Gliomas, the most common type of primary tumors of the central nervous system (CNS), account for almost 80% of brain malignancies, being glioblastoma (GBM) the most aggressive type[1-3]. Despite several advances in the field of neuro-oncology and the use of a multimodal treatment (surgery, radiotherapy and chemotherapy), the median survival for GBM patients remains extremely poor (~15 months)[4, 5].

Recent evidences suggest that tumor heterogeneity, and poor response and resistance to current therapies are partly due to the existence of cancer stem cells (CSCs). These cells share important characteristics with normal stem cells, including self-renewal, maintained proliferation, multipotency capacity, and have also been associated with therapy resistance and tumor initiation, progression, and recurrence[6]. CSCs have been isolated and characterized from several types of cancers, including gliomas. Human brain tumor stem cells (BTSCs) were isolated from primary tumors by cell sorting based on CD133 membrane expression[7, 8]. Functionally, CD133$^+$-tumor cells were considered to be CSCs as they generated non-adherent neurospheres, presented self-renewal and a high proliferation potential, and were multipotent[7]. It was also proved that CD133$^+$-BTSCs displayed *in vivo* tumorigenicity in immunocompromised mice, providing strong evidence for the crucial roles of CSCs in brain tumors[8]. Several subsequent studies corroborated the existence of BTSCs, as well as their increased resistance to radiotherapy and chemotherapy, as temozolomide (TMZ)[9-12].

Due to the high heterogeneity of GBMs, additional cell surface markers besides CD133[7, 13, 14] have been proposed to identify GBM stem cells (GSCs), including CD15 (SSEA-1)[15-17], A2B5[18-20], CD90[21], L1CAM[22, 23], CXCR4[24, 25] and the combination of CD44 and ID1[26]. However, these markers bear some caveats since not only their expression seems valid only for subset of GBMs and can change depending on environmental conditions[27], but their expression is not exclusive of GSCs[28-31]. Indeed, no generally accepted universal marker for highly malignant GSCs has been defined yet. In this way, it is critical to identify GSC-specific markers that can be used for the identification/isolation of this subpopulation. Thus, alternative identification and isolation methods based on functional properties of GSCs would avoid the use of artifact-prone surface markers.

Recently, Miranda-Lorenzo and colleagues have identified an intrinsic autofluorescent phenotype in CSCs derived from different human epithelial solid tumors, such as pancreatic ductal adenocarcinoma, colorectal carcinoma, hepatocellular carcinoma and non-small-cell lung carcinoma[32]. They showed that these autofluorescent cells could be identified and isolated by flow cytometry, and had features of CSCs, such as, were enriched in spheres culture and during chemotherapy, expressed pluripotency-associated genes, and showed tumorigenicity and invasiveness *in viv* [32]. Therefore, taking into account that this phenotype has not yet been studied in GBMs and that carcinomas and GBMs are very different types of tumors, we aimed to investigate if autofluorescence can be a new biomarker to improve GSCs identification, isolation and characterization.

## 3.2 Materials and Methods

### 3.2.1 Cell Culture

Seven different GBM cell line models were used: two commercially-available human GBM cell lines (U373 and U251), and 5 human primary GBM cell lines (GBM-1, GBM-12, GBM-18, GBM-19, and GBM-42) established in our lab as previously described[33]. Written informed consent was obtained from all patients.

GBM-1, GBM-12, GBM-19, and GBM-42 cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Biochrom) and GBM-18, U373 and U251 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Biochrom). All cell lines were supplemented with 10% fetal bovine serum (FBS; Biochrom) and 1% penicillin and streptomycin (Pen/Strep; Gibco), and were incubated at 37°C in a humidified atmosphere containing 5% (v/v) $CO_2$.

### 3.2.2 Flow cytometry analysis

Human primary GBM cell lines and single cells obtained from neurospheres dissociation were resuspended in FACS flow buffer (BD Bioscience) with DAPI (for exclusion of dead cells; 1:1000) before flow cytometry analysis using FACS Canto II (BD Biosciences). In order to identify autofluorescent ($Fluo^+$) cells, GBM cells were excited with a 488 nm blue laser and selected as the intersection with filters 530/40 and 580/30 (Supplementary Figure 3.1A).

To characterize autofluorescent cells, human primary GBM cells (GBM-1 GBM-18 and GBM-42) were analyzed by flow cytometry for the expression of CSC surface markers. Briefly, $1x10^5$ cells were incubated with the suitable dilution of appropriate isotype-matched control or specific antibody in 100 µL of PBS for 30 min at 4°C in the dark. Antibodies used were anti-CD133/1 (1:10; Miltenyi Biotec), anti-CD15 (1:10; BD Bioscience) and anti-CXCR4 (1:10; BD Bioscience). All antibodies were APC-conjugated. Cells were resuspended with 200 µL of FACS flow buffer (BD Bioscience) with DAPI and analyzed by FACS Canto II (BD Bioscience). Data was analyzed with FlowJo 10.0 software.

### 3.2.3 Cell Sorting

Before sorting, human primary and commercially available GBM cell lines were incubated overnight with 40 µM of Riboflavin (RBF; Sigma) in a humidified atmosphere at 37°C and 5% (v/v) $CO_2$.

Highly autofluorescent cell fraction ($Fluo^+$) and non-autofluorescent cells ($Fluo^-$) of human GBM cell lines were sorted using a FACS Aria III equipment (BD Biosciences) and correspondent data was analyzed by FACS Diva 7 software (BD Biosciences). Before cell sorting, cell lines were resuspended at a concentration of $5x10^6$ cells/mL in sorting buffer [PBS 1x; 3% FBS (v/v); 3 mM EDTA (v/v)] and filtered through a 40 µm strainer (BD Biosciences) to eliminate cell clumps. Cells were then sorted through a 100 µm nozzle at a sheath pressure of 20 psi. A yield sorting modality (Yield mask sorting for FACS Aria III) was chosen. Gating strategy for sorting was performed as indicated in Supplementary Figure 3.1B. To obtain high purity during sorting procedures an appropriate distance between gates for $Fluo^+$ and $Fluo^-$ cells is required. Sorted cells ($Fluo^+$ and $Fluo^-$ subpopulations) were collected in 5 mL polypropylene tubes (BD Biosciences) containing 1 mL collection medium (DMEM or RPMI supplemented with 20% FBS) and transferred to cell culture flasks with pre-warmed media (DMEM or RPMI supplemented with 10% FBS and 2% Pen/Strep).

### 3.2.4 Neurosphere formation assay

Neurospheres were generated by culturing 1500 human primary GBM cells in NeuroCult NS-A Proliferation Kit (Life Technologies) supplemented with 20 ng/mL epidermal growth factor (EGF; Invitrogen), 20 ng/mL basic fibroblast growth factor (b-FGF; Invitrogen) and 1% B27 (Invitrogen) in 24-multi well plates (0.5 mL/well). Cells were incubated for up to 21 days in a humidified atmosphere at 37°C and 5% (v/v) $CO_2$. Neurospheres were supplemented with fresh media every 4 days (250 µL/well). The number of neurospheres were counted after 21 days and pictures were taken. For serial passaging, neurospheres were harvested and dissociated with Accutase (EMD Millipore), every 21 days. The content of $Fluo^+$ cells in neurospheres was evaluated by flow cytometry as described before in 2.2 section from materials and methods.

### 3.2.5 Limiting Dilution Assay (LDA)

Cell number was adjusted to a starting concentration of $40x10^3$ cells/mL from which multiple serial dilutions were performed and plated in 96-well plates. At the end, cell densities ranged from 1000 to 1 cells per well in a final volume of 100 µl. Cells were cultured in the media used in the neurospheres and maintained in a humidified atmosphere at 37°C and 5% (v/v) $CO_2$. Cultures were supplemented with fresh media every 4 days. After 21 days, the fraction of wells not containing neurospheres was obtained for each condition and plotted against the initially plated cellular density. Stem cell frequencies and statistical significance was calculated using the ELDA software (available at http://bioinf.wehi.edu.au/software/elda/).

### 3.2.6 Temozolomide (TMZ) and Radiation treatment

For TMZ treatment, GBM cell lines (GBM-1, GBM-18, GBM-19 and GBM-42) were plated in T25 $cm^2$ flasks at an initial density of $1.5\times10^5$ and treated with the corresponding half-maximal inhibitory concentration ($IC_{50}$) of TMZ (600, 500, 850 and 400 μM, respectively) or vehicle (1% DMSO) for 9 days. The $IC_{50}$ was previously determined in the lab (data not shown). Culture medium containing TMZ or vehicle was renewed every 3 days. At each timepoint (3, 6 and 9 days), total cells were trypsinized and the percentage of $Fluo^+$ cells was evaluated by flow cytometry as described above.

After exposure to TMZ, FACS-sorted $Fluo^-$ and $Fluo^+$ cells viability was determined by MTS (Promega). $Fluo^-$ and $Fluo^+$ cells from human primary GBM cell lines GBM-1, GBM-18 and GBM-42 were plated at an initial density of 2500 cells/well in 48-multiwell plates, in duplicate, and incubated in a humidified atmosphere at 37°C and 5% (v/v) $CO_2$. After 3 days, to allow the cells to recover from sorting, both $Fluo^-$ and $Fluo^+$ GBM cells were treated with 600, 650 and 400 μM of TMZ (GBM-1, GBM-18 and GBM-42, respectively) or vehicle (1% DMSO) for 6 days. Culture medium containing TMZ or vehicle was renewed every 3 days. After the incubation period, cells were exposed to medium containing MTS in a 5:1 ratio for 2 hours in a humidified atmosphere at 37°C and 5% $CO_2$, and the optical density was determined at 490 nm.

For irradiation treatment, $1.5\times10^5$ cells of GBM-1, GBM-18, GBM-19 and GBM-42 cells were plated in 3.5 cm diameter plates, and were irradiated with 2, 4, 6, 8 and 10 Gy at 1.94 Gy/min, at room temperature in a 137Cs irradiator (Shepherd Mark-I [model SN1068]; J. L. Shepherd and Assoc., San Fernando, CA). Subsequently, cells were washed once with PBS and fresh media was added to the plates that were maintained in a humidified atmosphere at 37°C and 5% (v/v) $CO_2$. After three days, total cells were trypsinized and the percentage of $Fluo^+$ was evaluated by flow cytometry as described above.

### 3.2.7 RNA extraction and qRT-PCR

Total RNA from FACS-sorted human primary GBM cell lines was extracted with Trizol (Invitrogen) according to the manufacturer's instructions. cDNA synthesis was performed using 1 µg of total RNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene-specific mRNA levels were assessed by quantitative real-time PCR (qPCR) in a real-time thermocycler (CFX96; Bio-Rad) using Fast SYBR Green (Qiagen) according to the manufacturer's instructions, by the $2^{\Delta\Delta Ct}$ method. The list of primers used can be found in Supplementary Table 3.1.

### 3.2.8 Riboflavin (RBF), fumitremorgin C (FTC) and basal medium treatments

RBF and FTC treatments: GBM cell lines were plated at an initial density of $3x10^5$ cells/well in 6-multiwell plates, in duplicate, and incubated in a humidified atmosphere at 37°C and 5% (v/v) $CO_2$. After 24 hours, fresh media (control condition) or fresh media containing either RBF (40 µM) or FTC (5 µg/mL) was added to the respective wells.

Basal medium treatment: $4x10^5$ GBM cells were plated in 6-multiwell plates, in duplicate, and incubated in a humidified atmosphere at 37°C and 5% (v/v) $CO_2$. After 24 hours, cells were washed twice with PBS and fresh media (control condition), basal media (medium without RBF) or basal media containing RBF (40 µM) was added to the respective wells.

In all the assays, after 3 days of incubation, total GBM cells were trypsinized and washed twice with PBS and the content of $Fluo^+$ cells was evaluated by flow cytometry.

### 3.2.9 *In vivo* intracranial orthotopic GBM model

All experiments with mice were approved by institutional and national ethical committees (Direção Geral de Alimentação e Veterinária, Portugal) and in accordance with European Union Directive 2010/63/EU. Human commercially available U373 GBM cells were sorted into $Fluo^-$ and $Fluo^+$ cells using FACS Aria III equipment (BD Biosciences). A total of $5\times10^5$ cells (U373 $Fluo^-$ and $Fluo^+$) were stereotactically injected into the brain striatum (1.8 mm right, 0.1 mm front, and 2.5 mm deep from the bregma) of 12-weeks-old NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) male mice (6 mice per group). Animals' body weight was evaluated 3 times per week, and general behavior and symptomatology daily. Humane endpoints for sacrifice were established as severe weight loss (> 30% of their total body weight relative to the highest body weight value), neurological dysfunction, seizures or moribund condition. All brains were collected for histological and molecular analyses.

### 3.2.10 Immunohistochemistry

Tissues sections were deparaffinized and rehydrated by xylene and ethanol series. Sodium citrate buffer (10 mM, 0.05% Tween 20, pH 6) was used for antigen retrieval. Endogenous peroxidase activity was blocked with 3% $H_2O_2$ in TBS for 10 minutes. Ki-67 (#550609, BD Biosciences; 1:200), Nestin (#MAB5326, EMD Millipore; 1:100) and SOX2 (#AB5603, EMD Millipore; 1:500) immunohistochemical staining was performed based on the streptavidin-biotin-peroxidase complex principle using the LabVision kit (UltraVision Large Volume Detection System Anti-polyvalent, HRP) according to the manufacturer's instructions. Regarding Ki-67 staining, tissues were permeabilized using TBS-Tween 0.5%, for 10 minutes, before antigen retrieval. For all staining DAB substrate (DAKO) was used as chromogen, followed by counterstaining with hematoxylin.

### 3.2.11 Statistical analyses

All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad software, Inc.). To assess the statistical differences between groups in the *in vitro* assays, unpaired Student's *t*-test analysis was used. Overall survival of orthotopic GBM xenografted mice was compared between groups ($Fluo^-$ *vs.* $Fluo^+$) by the log-rank test and plotted as Kaplan-Meier curves. Results are presented as normalized means ± standard deviations (SD), and statistical significance was defined as $p \leq 0.05$ for a 95% confidence interval.

## 3.3 Results

### 3.3.1 Identification of autofluorescent cells in primary GBM cell lines

In order to investigate autofluorescence as a new marker to identify GSCs, we started by analyzing the presence of autofluorescent ($Fluo^+$) cells in 2 human primary GBM cell lines, GBM-18 and GBM-1, cultured in adherent conditions. Using confocal microscopy, both cell lines presented a fraction of cells with a green fluorescent vesicle (Figure 3.1A, arrows). Additionally, a panel of 5 human primary GBM cell lines (GBM-1, GBM-12, GBM-18, GBM-19, and GBM-42), in adherent conditions, were investigated for the presence of $Fluo^+$ cells by flow cytometry analysis. All GBM cell lines presented a small percentage of $Fluo^+$ cells when cultured in adherent conditions, ranging from 1.27% ± 0.13 to 4.59% ± 1.11 (Figure 3.1B). Growing these cells in stem cell conditions as neurospheres increased the percentage of $Fluo^+$ cells (Figure 3.1C and D), suggesting that autofluorescence could be a biomarker of GSCs.

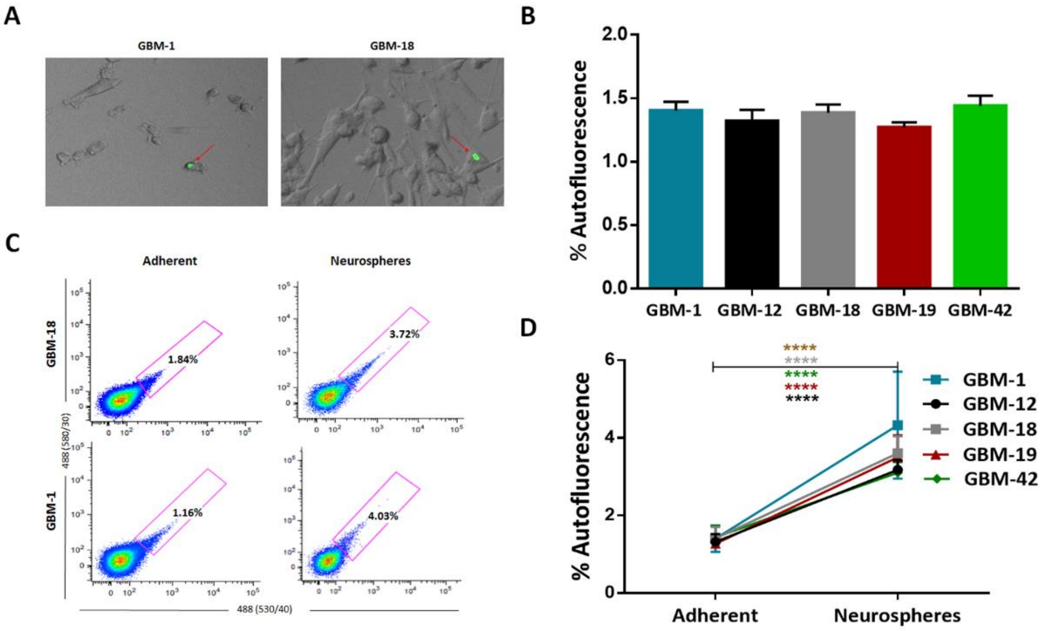

**Figure 3.1: Primary GBM cell lines present autofluorescent cells in adherent and neurospheres conditions. A)** Representative images of Autofluorescent (Fluo$^+$) cells (arrows) in human primary GBM cell lines, GBM-18 and GBM-1 grown in adherent conditions. **B)** Quantification, by flow cytometry, of the percentage of Fluo$^+$ cells across 5 different human primary GBM cell lines cultured in adherent conditions. **C)** Representative flow cytometry plots of the human primary GBM cell lines, GBM-18 and GBM-1 cultured in adherent and neurospheres conditions. **D)** Percentage of autofluorescence across 5 human primary GBM cell lines cultured as adherent cells or neurospheres ($n \geq 3$). Data is represented as the mean ± SD of at least three independent experiments ($*p \leq 0.05$, $**p \leq 0.01$, $****p \leq 0.0001$).

### 3.3.2 Autofluorescent cells present characteristics of GBM stem cells

In order to determine if these GBM autofluorescent cells displayed additional features of GSCs the expression of pluripotent/stem cell markers was evaluated. FACS-sorted Fluo$^+$ cells from 3 human primary GBM cell lines (GBM-18, GBM-1 and GBM-42) had significantly increased expression of a variety of pluripotency-associated genes, including *Bmi1*, *Klf4*, *Nanog*, *Nestin*, *Oct3/4* and *SOX2*, which are frequently overexpressed in cancer stem cells[34-37] comparing with their Fluo$^-$ counterparts cells (Figure 3.2A). Additionally, GSCs surface markers, such as CD133, CD15 and CXCR4 were increased in Fluo$^+$ cells compared to Fluo$^-$ cells (Figure 3.2B; Supplementary Figure 3.2).

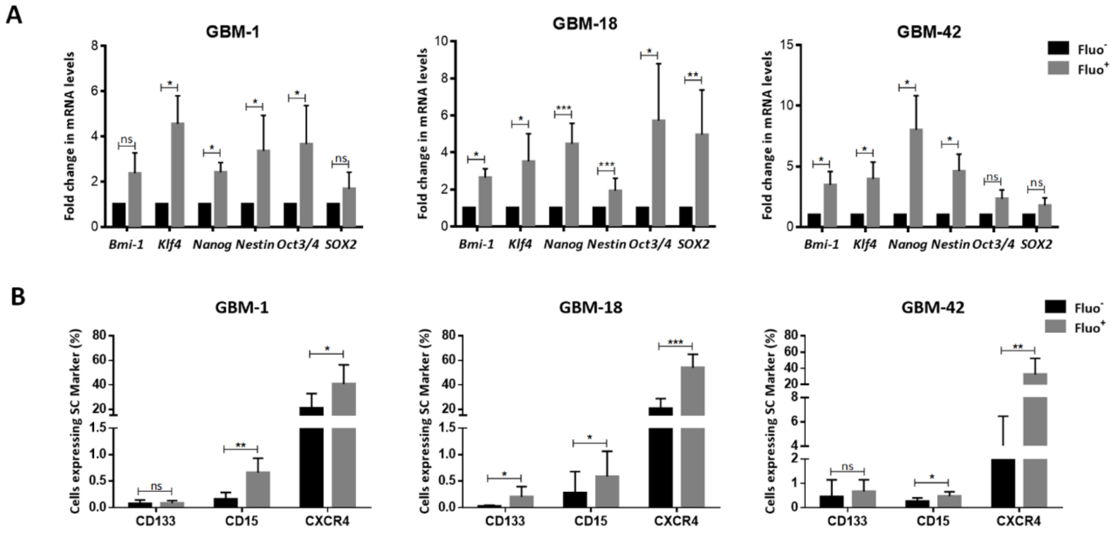

**Figure 3.2: Autofluorescent GBM cells have increased expression of stemness- and pluripotency-associated markers. A)** RT-qPCR analysis of pluripotency-associated genes (*Bmi-1, Klf4, Nanog, Nestin*, *Oct3/4* and *SOX2*) in FACS-sorted Fluo$^+$ and Fluo$^-$ cells from human primary GBM cell lines, GBM-18, GBM-1 and GBM-42. Data shown are normalized for *TBP* expression and represent the relative ratio between Fluo$^+$ *vs.* Fluo$^-$ ($n \geq 3$). **B)** Quantification of flow cytometry analysis for the indicated cell surface stem cell markers (CD133, CD15 and CXCR4) in human primary GBM cell lines, GBM-18, GBM-1 and GBM-42. Fluo$^+$ cells overexpressed stem cell markers at the protein level comparing with Fluo$^-$ subpopulation ($n \geq 3$). All data is represented as the mean ± SD of at least three independent experiments (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

Since clonogenic growth as neurospheres is linked to GBM stemness and is an *in vitro* indicator of self-renewal ability in GSCs [7, 38], we sorted Fluo$^-$ and Fluo$^+$ cells from GBM-18, GBM-1 and GBM-42 cell lines and evaluated the capacity of these cells to form neurospheres *in vitro*. Fluo$^+$ cells significantly increased the number of neurospheres compared to their Fluo$^-$ counterparts (Figure 3.3A), in all tested primary GBM cells. Additionally, self-renewal capacity was measured over three consecutive passages in GBM-18 and GBM-1 cells grown under neurospheres conditions, demonstrating that Fluo$^+$ cells formed significantly more neurospheres in all passages than Fluo$^-$ cells (Figures 3.3B and C). Similarly, LDA was carried out in FACS-sorted Fluo$^-$ and Fluo$^+$ from GBM-18 and GBM-1 cells. Concordantly, Fluo$^+$ cells displayed a higher frequency of neurosphere formation compared to Fluo$^-$ counterparts (1/(stem cell frequency): 1/8.32 for GBM-18 Fluo$^-$, 1/1.91 for GBM-18 Fluo$^+$, 1/13.4 for GBM-1 Fluo$^-$, and 1/5.81 for GBM-1 Fluo$^+$) (Figure 3.3D).

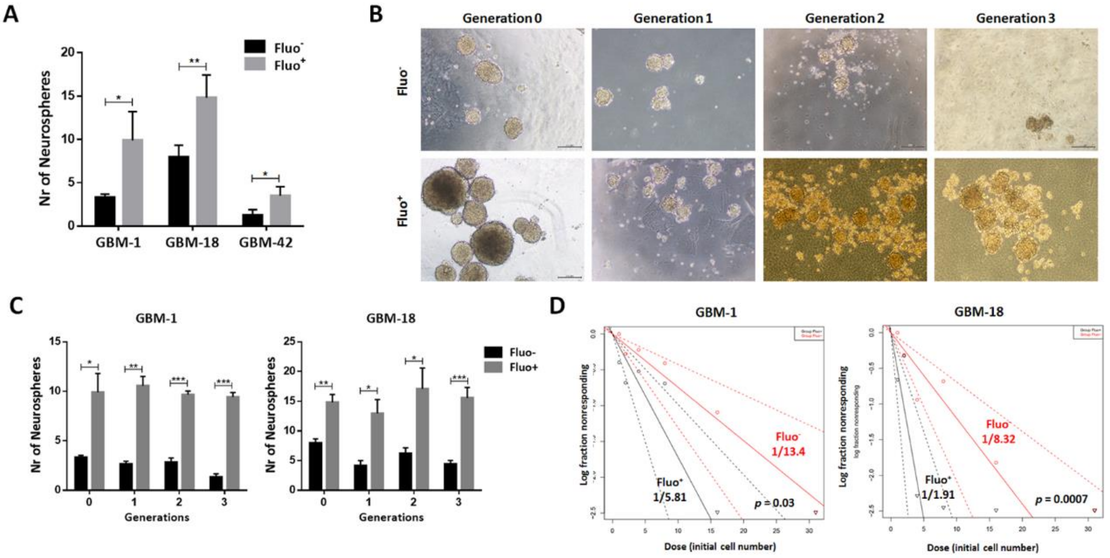

**Figure 3.3: Autofluorescent cells have a higher self-renewal ability. A)** Quantification of Fluo$^+$ and Fluo$^-$ neurospheres number from GBM-18, GBM-1 and GBM-42 sorted cells ($n \geq 3$; each performed in triplicate. **B)** Representative phase contrast photographs of GBM-18 Fluo$^+$ and Fluo$^-$ neurospheres over three generations. **C)** Quantification of neurospheres number of Fluo$^+$ and Fluo$^-$ sorted cells from human primary GBM cell lines GBM-18 and GBM-1, after three consecutive passages (generations) (n = 3, each performed at least in triplicate). **D)** Representative ELDA analysis of GBM-18 and GBM-1 Fluo$^+$ and Fluo$^-$ sorted cells (n = 2). Data is representative of at least three independent experiments ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$).

It has been described that GSCs are particularly resistant to chemo- and radio-therapy[9, 11, 12, 39, 40]. In line with this hypothesis, we treated human primary GBM cell lines, GBM-1, GBM-18, GBM-19 and GBM-42 with the $IC_{50}$ TMZ for each cell line (600, 500, 850 and 400 µM, respectively). The content of Fluo$^+$ cells was analyzed by flow cytometry after 3, 6 and 9 days of TMZ treatment. TMZ treatment led to a significant increase in the percentage of Fluo$^+$ cells, in all primary GBM cell lines, in a time-dependent manner (Figure 3.4A and Supplementary Figure 3.3A). Additionally, the same human primary GBM cell lines were exposed to increased doses of radiation (0, 2, 4, 6, 8 and 10 Gy). Radiation treatment increased the percentage of GBM Fluo$^+$ cells in a dose-dependent manner (Figure 3.4B and Supplementary Figure 3.3B).

Moreover, cell viability of FACS-sorted Fluo$^-$ and Fluo$^+$ cells from human primary GBM cell lines (GBM-18, GBM-1 and GBM-42) was determined after 6 days of TMZ treatment. Regarding GBM-18 and GBM-1 cell lines, no significant differences on cell viability were found between Fluo$^-$ and Fluo$^+$ cells (Supplementary Figure 3.3C). Contrarily, FACS-sorted GBM-42 Fluo$^+$ cells presented a statistically significant increase on cellular viability after TMZ treatment compared to Fluo$^-$ cells (Supplementary Figure 3.3C).

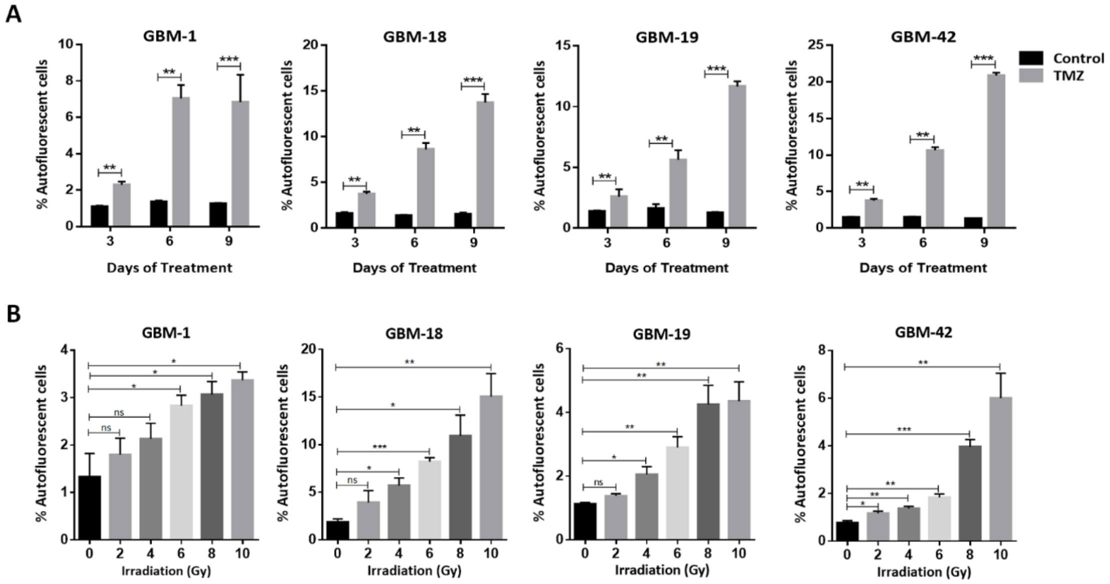

**Figure 3.4: Temozolomide and radiation treatments increase the percentage of GBM autofluorescent cells. A-B)** Quantification of Fluo$^+$ percentage in control *vs.* Temozolomide (TMZ)-treated GBM cells **(A)** and in control *vs.* irradiated cells (2, 4, 6, 8, and 10 Gy) **(B).** Data is represented as the mean ± SD of three independent experiments ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$).

Altogether, our data demonstrate that the population of Fluo$^+$ cells in GBM presents functional GSCs features, displaying increased expression of pluripotency-associated genes and stem cell markers, enriched capacity to grow as neurospheres, a higher self-renewal ability, and their proportion was increased after chemo- and radiotherapy treatment.

### 3.3.3 Riboflavin as the source of autofluorescence

Previously, Miranda-Lonrenzo and colleagues showed that the fluorescent vitamin riboflavin (RBF; vitamin B2) was the substrate, used by ABCG2 transporters, responsible for the autofluorescent phenotype in CSCs from carcinomas[32]. Therefore, we tested in human primary (GBM-1, GBM-12, GBM-18, GBM-19, and GBM-42) and commercially available (U373 and U251) GBM cell lines if RBF levels were associated with the autofluorescent phenotype observed in GBM. We observed that treatment with RBF led to a significant increase in the content of Fluo$^+$ in all GBM cell lines (Figure 3.5A and B and Supplementary Figure 4.4A). Additionally, GBM cell lines were cultured in basal media (medium without RBF) resulting in a significant decrease in the percentage of Fluo$^+$ (Figure 3.5C and D and Supplementary Figure 4.4B). This was reversed when RBF was added to basal media (Figure 3.5C and D and Supplementary Figure 4.4B). By RT-qPCR we observed that *ABCG2* was significantly overexpressed in the Fluo$^+$ subpopulation compared with the negative counterparts

(Fluo$^-$) in the human primary GBM cell lines GBM-18, GBM-1 and GBM-42 (Figure 3.5E). Finally, and taking into account that fumitremorgin C (FTC) is a recognized inhibitor of ABCG2 transporting activity - by allosteric binding to the protein that causes a conformational change that results in the impairment of ABCG2-mediated transport - GBM cells were treated with this drug. The functional inhibition of ABCG2 with FTC significantly decreased the percentage of Fluo$^+$ cells in all primary GBM cell lines tested (Figure 3.5F and G). Together, these data suggest that autofluorescence was a result of the accumulation of riboflavin in GSCs and that ABCG2 is involved in the transport of this vitamin.

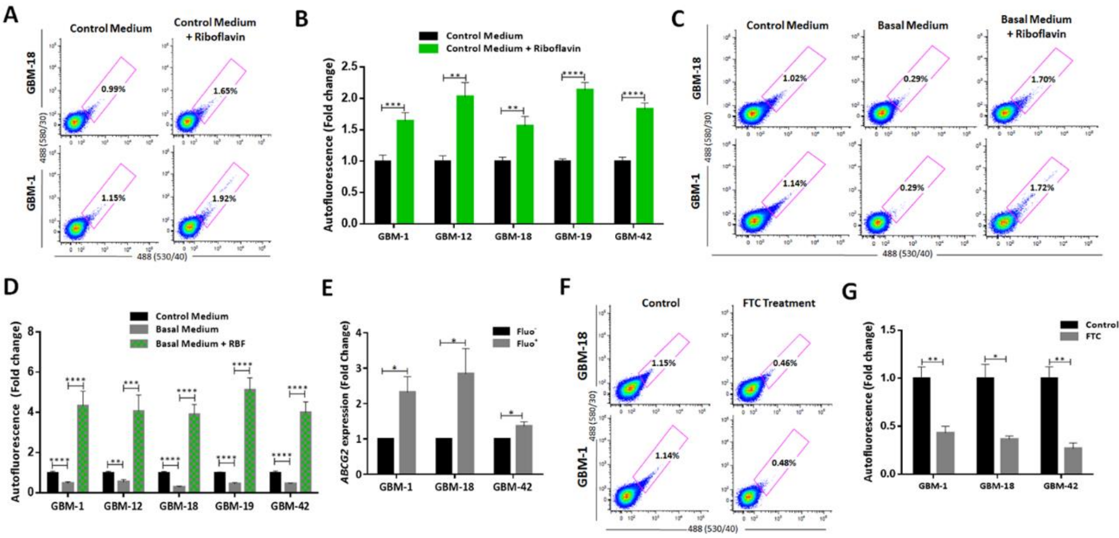

**Figure 3.5: Riboflavin as the source of autofluorescence of glioblastoma stem cells**. **A-B)** Representative flow cytometry analysis **(A)** and quantification **(B)** of Fluo$^+$ cells in GBM-18 and GBM-1 cultured in control medium or control medium supplemented with 40 μM of Riboflavin (RBF) during three days. **C-D)** Representative flow cytometry plots **(C)** and quantification **(D)** of Fluo$^+$ cells in GBM-18 and GBM-1 cells cultured in adherent conditions in control medium, basal medium or basal medium supplemented with 40 μM of RBF after three days in culture. **E)** RT-qPCR analysis of *ABCG2* transporter in Fluo$^+$ and Fluo$^-$ sorted cells from GBM-18, GBM-1 and GBM-42 cell lines. Data is normalized for *TBP* expression and represent the relative ratio between Fluo$^+$ $_{\text{vs.}}$ Fluo$^-$. **F-G)** Representative flow cytometry analysis **(F)** and quantification **(G)** of autofluorescence in GBM-18 and GBM-1 cells treated with fumitremorgin C (FTC) during 72h at 5 mg/mL. Data from B, D, E and G panels is represented as the mean ± SD of at least three independent experiments, while A, C and F panels are representative plots of at least three independent experiments ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ $****p \leq 0.0001$).

### 3.3.4 Autofluorescence GSCs are associated with shorter survival in orthotropic GBM xenografts models

In order to determine whether the subpopulation of Fluo$^+$ cells present a more aggressive phenotype *in vivo*, the commonly used orthotopic U373 human GBM model was used[10, 41, 42].

FACS-sorted U373 Fluo$^-$ and Fluo$^+$ cells were orthotopically injected into the brain striatum of NSG male mice. Animals bearing U373 Fluo$^+$-derived tumors presented significantly lower overall survival (median 70 days) than their respective Fluo$^-$ counterparts (median 99 days; Log rank test, $p$ = 0.039; Figure 3.6A). Hematoxylin & Eosin (H&E) analyses confirmed tumor formation in mice brains that display characteristic hallmarks of GBMs, such as pleomorphic and spindle shape tumor cells, high mitotic activity and prominent nuclear polymorphism (Figure 3.6B). Additionally, tumors derived from U373 Fluo$^+$ cells presented an increased number of Ki-67-positive cells, as well as increased expression of Nestin and Sox2, when compared to Fluo$^-$ tumors (Figure 3.6C).

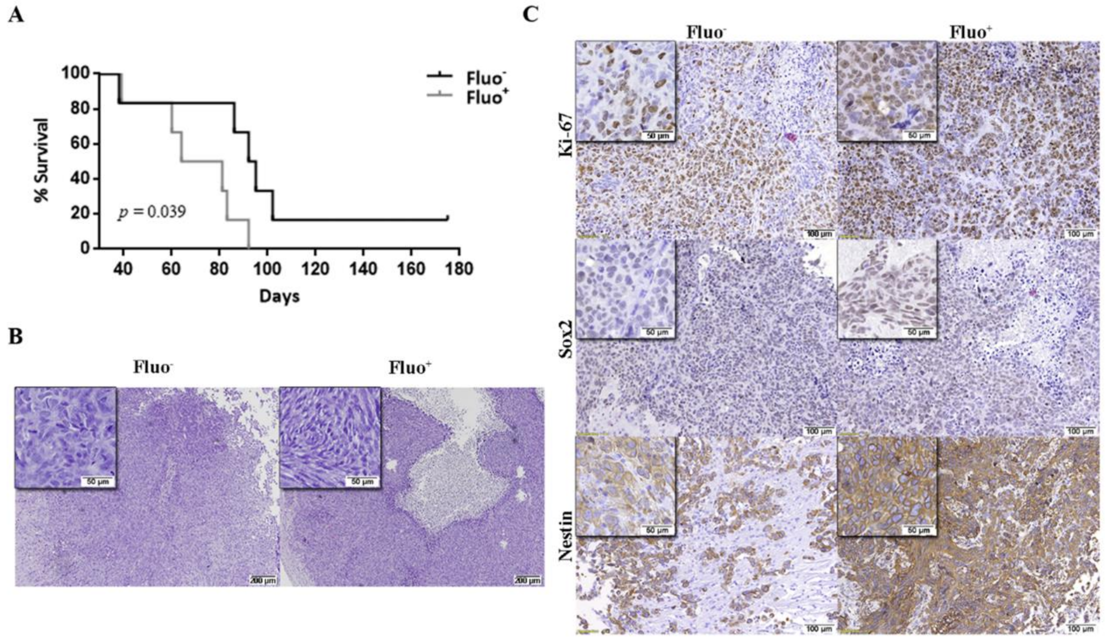

**Figure 3.6: Autofluorescent cells are associated with increased aggressiveness in *in vivo* intracranial orthotopic GBM xenografts. A)** Fluo$^+$ and Fluo$^-$ cells from U373 GBM cell line were intracranially injected into NSG male mice (6/group). Kaplan-Meier survival curves of NSG mice injected with U373 Fluo$^-$ and Fluo$^+$ sorted cells (Log-rank test, $p$ = 0.039). **B-C)** Representative images of hematoxylin and eosin **(B)** and Ki-67, SOX2 and Nestin **(C)** staining in tissue sections of mice brains orthotopically injected with U373 Fluo$^-$ and Fluo$^+$ sorted cells.

## 3.4 Discussion

In this work, we identified an intrinsic autofluorescent phenotype in GBM cells that is associated with GSCs in several human primary GBM cell lines, independently of the expression of common cell surface stem cell markers. We also showed that the underlying mechanism of the increased autofluorescent phenotype was the accumulation of riboflavin in cytoplasmic vesicles in GSCs. These Fluo$^+$ cells could be identified by flow cytometry and isolated by FACS from human primary and commercially available GBM cell lines without the need of antibodies.

GSCs are defined by functional characteristics including persistent proliferation, self-renewal capacity, stem cell markers expression, differentiation into multiple lineages and tumor initiation and progression[43]. Interestingly, our data demonstrates that Fluo$^+$ cells possess some of these functional characteristics, including overexpression of stem and pluripotent-associated markers (Figure 3.2); increased self-renewal capacity (Figure 3.3); and were associated with shorter survival *in vivo* (Figure 3.6). Additionally, we demonstrated that treatment with TMZ and radiation increased the content of Fluo$^+$ cells (Figure 3.4 and Supplementary Figure 3.3). Therefore, we evaluated the viability of both Fluo$^+$ and Fluo$^-$ cells after TMZ treatment (Supplementary Figure 3.3C), observing that Fluo$^+$ cells from GBM-42 had an increased cell viability compared to Fluo$^-$ subpopulation (Supplementary Figure 3.3C), while the viability of Fluo$^+$ and Fluo$^-$ cells sorted from GBM-18 and GBM-1 after TMZ exposure (higher doses of TMZ compared to GBM-42 cell line) was similar (Supplementary Figure 3.3C). This might be due to TMZ-induced conversion of non-GSCs into GSCs cells, as previously demonstrated[44, 45]. Together, these results demonstrated that Fluo$^+$ cells possess defining and recognized GSCs features, strongly suggesting that this autofluorescent phenotype can be used to identify and isolate GSCs.

Additionally, we demonstrated that the autofluorescent phenotype was due to the accumulation of RBF inside GBM cells (Figure 3.5A-D and Supplementary Figure 4.4A and B), which was also previously showed by Miranda-Lourenzo and colleagues [32]. Moreover, we verified that Fluo$^+$ subpopulation overexpressed *ABCG2* compared to Fluo$^-$ cells (Figure 3.5E). This result is in line with previous studies that showed that ABCG2 is highly expressed in stem cells, including GSCs[46-49]. Previously, it was demonstrated that ABCG2 (i) secretes RBF (vitamin B2) into milk in the lactating mammary gland; (ii) is responsible for the uptake of this vitamin into cancer cells[50, 51]; and (iii) mediates a marked intravesicular concentration of RBF in ABCG2-overexpressing breast and lung cancer cells[51]. This is in agreement to Miranda-Lourenzo and colleagues' report showing that ABCG2 mediated the transport of RBF in CSCs[32]. Here, due to the role of ABCG2 in the uptake of RBF, we show that the pharmacological inhibition of ABCG2 pharmacological inhibition with FTC led to a significant decrease in the percentage of Fluo$^+$ cells (Figure 3.5F and G). However, this decrease was not complete, raising the possibility that RBF uptake might be performed by other transporters than ABCG2. In fact, Fu and colleagues revealed that riboflavin transporter 2 (RFT2), a human RBF transporter, is expressed in glioma cells, correlated with WHO grade, and has a role in glioma cells migration, invasion and proliferation[52]. Altogether these results demonstrated that the autofluorescent phenotype was in part due to an influx of RBF performed by ABCG2 transporter.

In the future, it will be important to clarify if RBF has a functional role in the biology of GSCs. In fact, it is known that RBF is involved in numerous enzymatic reactions in all forms of life and performs key metabolic functions by mediating the transfer of electrons in biological oxidation-reduction reaction, including bioenergetic metabolism[53, 54].

In conclusion, in this study, we show that autofluorescence can be used as marker to identify and isolate GSCs that will allow to overcome problems associated with the use of cell surface markers to more easily and specifically isolate GSCs, to search for new biomarkers by characterizing the cell surface proteome of this autofluorescent GSCs, and to develop new GSC-specific therapies.

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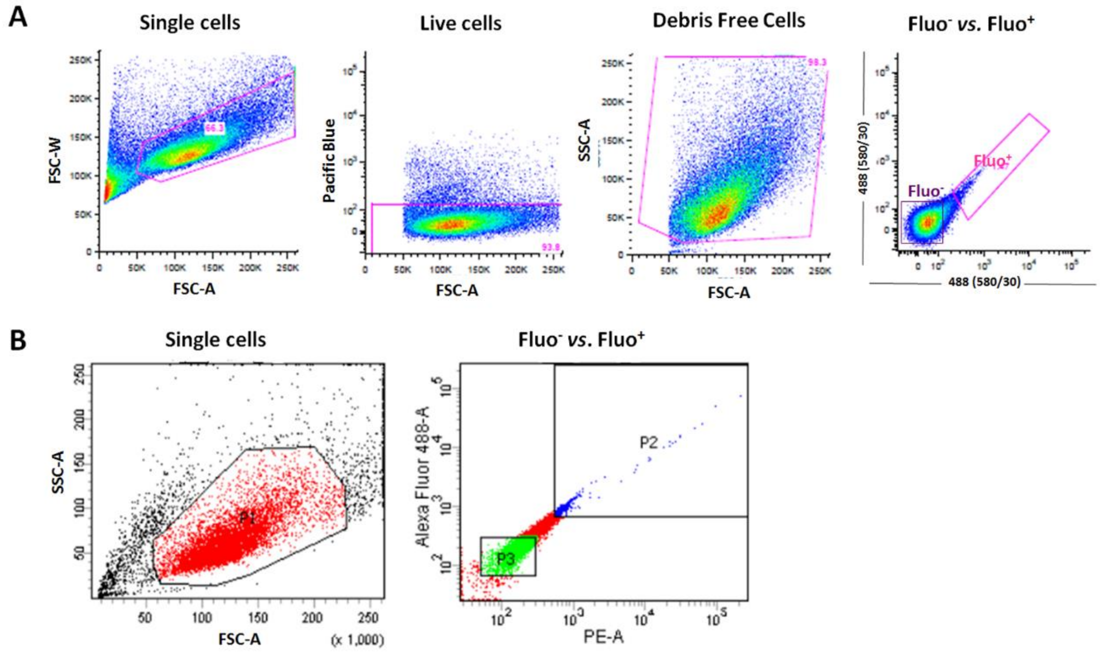
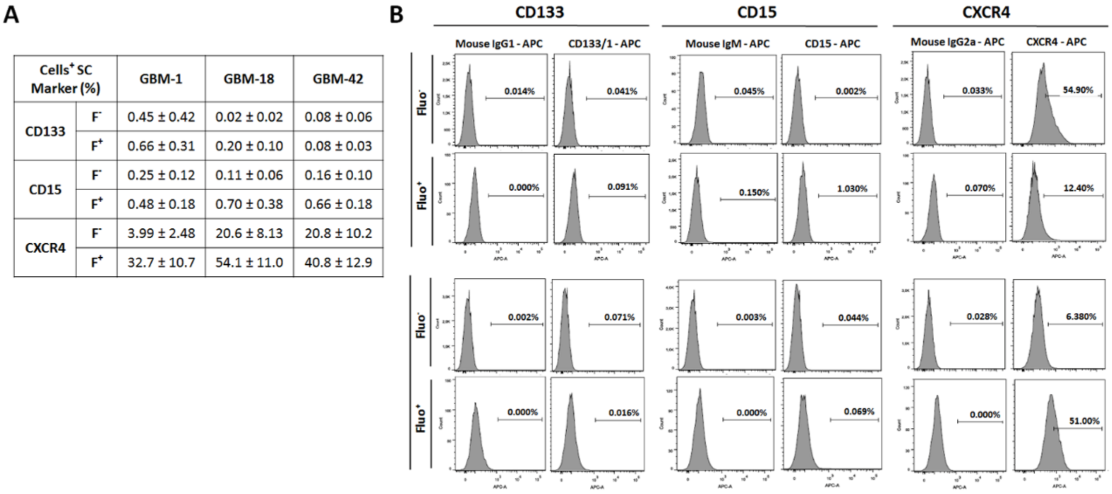
## 3.6 Supplementary Information

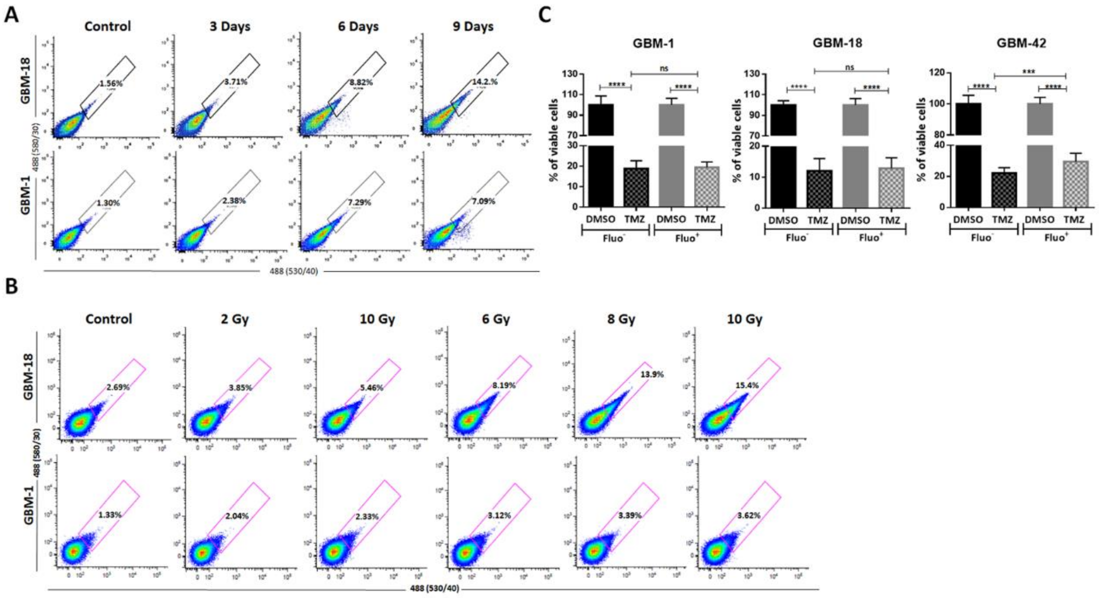


**Supplementary Figure 3.1: Identification of autofluorescent cells by flow cytometry**. **A)** Representative flow cytometry plots demonstrating the strategy used for the identification of Fluo$^+$ cells. These cells are excited with a 488-nm blue laser and selected with the intersection of 530/40 and 580/30 filters, where Fluo$^+$ corresponds to autofluorescent subpopulation and Fluo$^-$ corresponds to non-autofluorescent cells. **B)** Gating strategy used for sorting Fluo$^+$ and Fluo$^-$. A FITC *vs.* PE dot plot was performed, P2 corresponds to Fluo$^+$ fraction and P3 corresponds to Fluo$^-$ subpopulation.

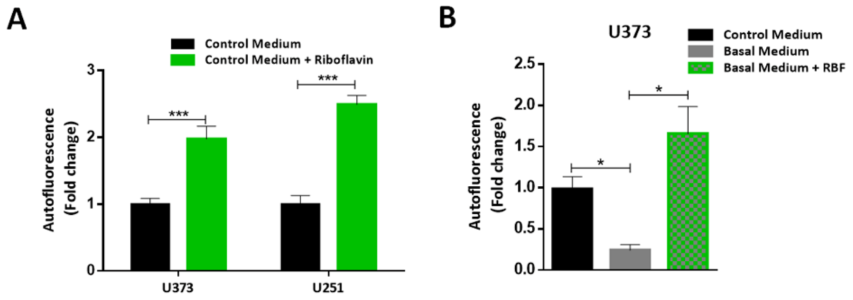


| Cells$^+$ SC Marker (%) | | GBM-1 | GBM-18 | GBM-42 |
|---|---|---|---|---|
| CD133 | F$^-$ | 0.45 ± 0.42 | 0.02 ± 0.02 | 0.08 ± 0.06 |
| | F$^+$ | 0.66 ± 0.31 | 0.20 ± 0.10 | 0.08 ± 0.03 |
| CD15 | F$^-$ | 0.25 ± 0.12 | 0.11 ± 0.06 | 0.16 ± 0.10 |
| | F$^+$ | 0.48 ± 0.18 | 0.70 ± 0.38 | 0.66 ± 0.18 |
| CXCR4 | F$^-$ | 3.99 ± 2.48 | 20.6 ± 8.13 | 20.8 ± 10.2 |
| | F$^+$ | 32.7 ± 10.7 | 54.1 ± 11.0 | 40.8 ± 12.9 |

**<u>Supplementary Figure 3.2</u>: Autofluorescent GBM cells have increased expression of stem cell surface markers. A)** Summary of CD133, CD15 and CXCR4 expression in different Fluo$^+$ and Fluo$^-$ cells derived from GBM-18, GBM-1 and GBM-42 cells. Data is represented as the mean ± SD of three independent experiments (*$p \leq 0.05$, ****$p \leq 0.0001$). **B)** Representative flow cytometry analysis for the indicated stem cell surface markers in Fluo$^+$ and Fluo$^-$ cells from GBM-18 cell line.



**Supplementary Figure 3.3: Autofluorescent cells are more resistant to therapy. A-B)** Representative images of autofluorescent cells in human primary GBM cell lines, GBM-18 and GBM-1, in control (DMSO) *vs.* TMZ-treated cells over time **(A)** and in control *vs.* irradiated cells (2, 4, 6, 8, and 10 Gy) **(B)**. **C)** Percentage of cell viability in Fluo$^{+}$ and Fluo$^{-}$ sorted cells from GBM-18, GBM-1 and GBM-42 cells after TMZ or DMSO treatment for 5 days (n = 3, each performed in duplicate). Data is represented as the mean ± SD of three independent experiments (***$p \le 0.001$, ****$p \le 0.0001$).



**Supplementary Figure 3.4: Riboflavin is the source of autofluorescent cells in commercially available GBM cell lines. A)** Quantification of autofluorescent cells in two commercially available GBM cell lines U251 and U373 cultured in control media (DMEM) or control media containing 40 μM of Riboflavin (RBF), during three days. **B)** Quantification of autofluorescent content in U373 GBM cell line cultured in control medium, basal medium or basal medium supplemented with 40 μM of RBF. Data is represented as the mean ± SD of three independent experiments ($*p \leq 0.05$, $***p \leq 0.001$).

**Supplementary Table 3.1**: Sequence of primers used for RT-qPCR analyses.

| **Gene** | **Primer Sense** | **Primer Antisense** |
|---|---|---|
| ***ABCG2*** | TCATGTTAGGATTGAAGCCAAAGGC | TGTGAGATTGACCAACAGACCTGA |
| ***Bmi1*** | TTCTTTGACCAGAACAGATTGG | GCATCACAGTCATTGCTGCT |
| ***Klf4*** | ACCCACACAGGTGAGAAACC | ATGTGTAAGGCGAGGTGGTC |
| ***Nanog*** | TGAACCTCAGCTACAAACAGGTG | AACTGCATGCAGGACTGCAGAG |
| ***Nestin*** | CAGGAGAAACAGGGCCTACA | TGGGAGCAAAGATCCAAGAC |
| ***Oct3/4*** | CTTGCTGCAGAAGTGGGTGGAGGAA | CTGCAGTGTGGGTTTCGGGCA |
| ***SOX2*** | AGAACCCCAAGATGCACAAC | CGGGGCCGGTATTTATAATC |
| ***TBP*** | GAGCTGTGATGTGAAGTTTCC | TCTGGGTTTGATCATTCTGTAG |

For all genes, qPCR parameters were as follows: 4 minutes at 94 °C, 40 cycles of denaturation for 30 seconds at 94 °C, annealing for 30 seconds at 60 °C, and extension at 72 °C for 30 seconds, and final extension at 72 °C for 8 minutes.

# Chapter 4: Impact of Mesenchymal Stem Cells' Secretome on Glioblastoma Pathophysiology

The results presented throughout this chapter are under review for publication in an international peer reviewed journal:

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# Impact of Mesenchymal Stem Cells' Secretome on Glioblastoma Pathophysiology

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**Conflict of Interest**

The authors disclose no potential conflicts of interest.

## Abstract

**Background:** Glioblastoma (GBM) is a highly aggressive primary brain cancer, for which curative therapies are not available. An emerging therapeutic approach suggested to have potential to target malignant gliomas has been based on the use of multipotent mesenchymal stem cells (MSCs), either unmodified or engineered to deliver anticancer therapeutic agents, as these cells present an intrinsic capacity to migrate towards malignant tumors. Nevertheless, it is still controversial whether this innate tropism of MSCs towards the tumor area is associated with cancer promotion or suppression. Considering that one of the major mechanisms by which MSCs interact with and modulate tumor cells is via secreted factors, we studied how the secretome of MSCs modulates critical hallmark features of GBM cells.

**Methods:** The effect of conditioned media (CM) from human umbilical cord perivascular cells (HUCPVCs, a MSC population present in the Wharton jelly of the umbilical cord) on GBM cell viability, migration, proliferation and sensitivity to temozolomide treatment of U251 and SNB-19 GBM cells was evaluated. The *in vivo* chicken chorioallantoic membrane (CAM) assay was used to evaluate the effect of HUCPVCs CM on tumor growth and angiogenesis. The secretome of HUCPVCs was characterized by proteomic analyses.

**Results:** We found that both tested GBM cell lines exposed to HUCPVCs CM presented significantly higher cellular viability, proliferation and migration. In contrast, resistance of GBM cells to temozolomide chemotherapy was not significantly affected by HUCPVCs CM. In the *in vivo* CAM assay, CM from HUCPVCs promoted U251 tumor cells growth, despite not affecting angiogenesis. Proteomic analysis to characterize the secretome of HUCPVCs identified several proteins involved in promotion of cell survival, proliferation and migration, revealing novel putative molecular mediators for the effects observed in GBM cells exposed to HUCPVCs CM.

**Conclusions:** These findings provide novel insights to better understand the interplay between GBM cells and MSCs, raising awareness to potential safety issues regarding the use of MSCs as stem-cell based therapies for GBM.

**Keywords:** Glioblastoma; Mesenchymal stem cells; Human umbilical cord perivascular cells; Conditioned media; Secretome; Viability; Proliferation; Migration; Proteomics

## 4.1 Introduction

Gliomas are the most common primary malignancies in the central nervous system (CNS), accounting for approximately 80% of all primary brain tumors[1]. Glioblastoma (GBM, grade IV) is the most common and malignant type of glioma in adults, presenting a high mortality rate and very poor patient outcomes. In fact, despite multimodal therapeutic approaches consisting of surgery, chemotherapy and radiotherapy, virtually all GBMs recur and lead to death, presenting a median overall survival of ~15 months[2] This poor outcome has not changed significantly in the last decades, stressing the need for novel therapeutic strategies that may, more efficiently, overcome the highly resistance nature of these tumors.

A novel therapeutic approach currently being investigated for a variety of cancer types is based on the use of tumor-trophic stem cells, such as mesenchymal stem cells (MSCs)[3-17].These are multipotent progenitor cells that are defined according to 3 main characteristics: 1) expression of CD105, CD73 and CD90 (MSCs markers), and lack of expression of CD45, CD34, CD14 (hematopoietic markers); 2) ability to adhere to plastic surfaces; and 3) differentiation capacity into adipocytes, osteoblasts and chondrocytes (multipotency)[18, 19]. Additionally, MSCs are also characterized by their proliferative and self-renewal abilities, and can be isolated from bone marrow (BM-MSCs)[20], adipose tissue (ASCs)[21], umbilical cord (*e.g.*, human umbilical cord perivascular cells; HUCPVCs)[22-24], among other sources[25-30]. The use of MSCs is relatively promising since these cells: i) can be easily isolated and subsequently expanded *in vitro*; ii) show multi-lineage differentiation ability; iii) have an immune privileged nature; iv) present capacity to home for site of injury, including tumors; and v) are amenable to genetic modification[31, 32]. In fact, it was already demonstrated that MSCs present an intrinsic capacity to migrate towards gliomas and present low immunogenicity at autologous transplantation[3, 10-12, 33-42]. However, whether this selective MSC tumor-tropism is associated with cancer suppression or promotion functions is still controversial[3, 10, 11, 13, 34, 35, 43]. Several studies, using MSCs engineered to express anti-glioma agents, demonstrated that these cells are highly effective as anti-tumor delivery agents[10, 11, 36, 38, 42, 44, 45]. However, few studies have evaluated the impact of non-engineered MSCs on glioma behavior[3, 12, 13, 46], demonstrating that MSCs can act either by inducing or repressing tumor cells behavior. Akimoto and colleagues showed that umbilical cord blood-derived MSCs induced apoptosis in glioma cells; however, in the same study, adipose-derived MSCs enhanced the growth of GBM cells[3]. In another study, co-culturing of adipose-derived MSCs with human glioma cells led to higher survival and proliferation of glioma cells[12], whereas in another study, bone marrow-derived MSCs co-cultured with human glioma cells inhibited tumor cell proliferation [46]. Therefore, further studies focusing on the crosstalk between tumor cells and MSCs should be performed to strengthen the evidence that MSCs-based therapies could be efficiently and safely translated into clinical settings.

This study evaluates how the secretome of a population of MSCs isolated from Wharton Jelly of the umbilical cord (HUCPVCs) modulates critical hallmark features of GBM. In particular, using *in vitro* and *in vivo* models, we investigated the effect of HUCPVCs conditioned media (CM) on GBM cells viability, growth, migration, proliferation, angiogenesis, and response to chemotherapy. Proteomic analysis of HUCPVCs CM was performed to identify molecular players that can influence the behavior of GBM cells, which may identify novel targets for therapy.

## 4.2 Materials and Methods

### 4.2.1 Cell lines

The human glioblastoma cell line SNB-19 was kindly provided by Rui M. Reis, (Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Portugal) and the human glioblastoma cell line U251 was kindly provided by Professor Joseph Costello, (Department of Neurological Surgery, University of California, San Francisco (UCSF), USA). Both cell lines were cultured as monolayers in Dulbecco's Modified Eagle Medium (DMEM; Biochrom) supplemented with 10% (v/v) inactivated fetal bovine serum (FBS; Biochrom), and 1% (v/v) penicillin/streptomycin (Invitrogen). The human umbilical cord perivascular cells (HUCPVCs) were kindly provided by Prof. John E. Davies (University of Toronto, Toronto, Canada; previous characterized by Teixeira and colleagues[47]) and were grown as monolayers in alpha-Minimum Essential Medium (α-MEM; Gibco®) supplemented with 10% FBS (Biochrom) and 1% penicillin/streptomycin (Invitrogen). All cell lines were incubated at 37 °C in a humidified atmosphere with 5% (v/v) $CO_2$.

### 4.2.2 HUCPVCs conditioned media (CM) collection

Conditioned media (CM) were collected from HUCPVCs in culture at passage 6 (P6). For the *in vitro* assays (N = 7), HUCPVCs were plated at a density of $4\times10^3$ cells/cm$^2$ and allowed to grow for 72 hours. Subsequently, cells were washed twice with phosphate buffer solution (PBS) and the medium was replaced by DMEM with 1 % penicillin/streptomycin. For proteomic analysis (N = 1), HUCPVCs were plated at a density of $12\times10^3$ cells/cm$^2$ and allowed to grow for 72 hours. Subsequently, cells were washed five times with PBS and twice with DMEM, and the medium was replaced by DMEM containing 1% penicillin/streptomycin. CM were collected after 48 hours (culture media was not renewed or added during this period), filtrated through 0.22 µm filters, immediately snap-frozen and stored at -80°C. Before use, CM from HUCPVCs were thawed overnight at 4°C and only for the *in vitro* assays, 1 % of FBS was added to the media (HUCPVCs CM). The control condition, for the *in vitro* studies, consisted of DMEM containing 1% FBS and 1% penicillin/streptomycin (control media). For proteomic analysis control media consisted in DMEM containing 1% penicillin/streptomycin.

### 4.2.3 Cell viability assessment

#### 4.2.3.1 Trypan blue

U251 and SNB-19 GBM cells were plated in triplicates, at an initial density of $5\times10^4$ and $1\times10^5$ cells per well in 24-well plates, respectively. After 24 hours, HUCPVCs CM or control media were added to cells and incubated for 2, 4, and 6 days (CM and control media were renewed every 48 hours). At each time point, total cells were trypsinized and the suspension mixed with trypan blue (1:1 ratio). The number of viable cells were counted in duplicates using hemocytometers. Results represent the mean ± standard deviations (SD) of at least three independent experiments.

#### 4.2.3.2 MTT assay

U251 and SNB-19 GBM cells were plated in triplicates, at an initial density of $2.5\times10^4$ and $5\times10^4$ cells per well in 48-well plates, respectively. After 24 hours, HUCPVCs CM or control media were added to cells and incubated for 2, 4, and 6 days (CM and control media were renewed every 48h). At each time point, a MTT solution (Thermo Scientific; 0.5 mg of MTT per 1 mL of PBS) was added to each well, followed by incubation in a humidified atmosphere, at 37°C and 5% (v/v) $CO_2$, for 1 hour. The optical density was measured at 570 nm using a microplate reader. Results are presented as the mean ± SD of at least three independent experiments.

### 4.2.4 Response to temozolomide chemotherapy

To evaluate the effect of HUCPVCs CM on the response of GBM cells to temozolomide (TMZ, Sigma-Aldrich, dissolved in DMSO), the half-maximal inhibitory concentration ($IC_{50}$) of TMZ on U251 and SNB-19 GBM cells was determined by MTT assay. U251 and SNB-19 GBM cells were plated in triplicates, at an initial density of $1\times10^4$ and $2\times10^4$ cells per well in 24-well plates, respectively. After 24 hours, cells were washed once with PBS and treated with different doses of TMZ (5, 10, 20, 35, 50, 100, 500 µM; or 25, 50, 100, 175, 250, 500, 1000 μM; for U251 and SNB-19 cells, respectively) or vehicle (1% DMSO) in HUCPVCs CM or control media for 5 days (medium with drugs or vehicle was renewed after 2 days). Results are presented as the mean ± SD of three independent experiments.

### 4.2.5 Migration (wound healing) assay

U251 and SNB-19 GBM cells were plated in triplicates, at an initial density of $5\times10^5$ and $2.5\times10^5$ cells/well in 12-well plates, respectively. After 24 hours, a confluent cell monolayer was formed, and a wound was made by manually scratching with a 200 µL pipette tip. Cells in suspension were removed, and adherent cells were washed once with PBS. HUCPVCs CM or control media were carefully added to cells. At this point (0 hours), the "wounded" areas were photographed at 4 distinct places, at 40 x magnification by phase contrast microscopy. The same areas were subsequently photographed to monitor wound closure after 16, 24 and 48 hours. Migration distances were measured using the beWound - Cell Migration Tool (Version 1.5) as previously described[48]. Relative wound closure was calculated for each time point. Results are presented as percentages of wound closure and represent the mean ± SD of at least three independent experiments.

### 4.2.6 Cell Proliferation assay

To evaluate the impact of HUCPVCs CM on GBM cells proliferation, the Cell Proliferation ELISA, 5-bromo-2´-deoxyuridine assay (BrdU, Cell Proliferation ELISA, Applied Sciences) was used as indicated by the manufacturer. Briefly, U251 and SNB-19 GBM cells were plated in triplicates, at an initial density of $1.5\times10^3$ and $2.5\times10^3$ cells per well, in 96-well plates, respectively, and grown overnight. Then, adherent cells were treated with HUCPVCs CM or control media for 3 days. After this period, cells were labeled with 10 μL/well of 100 μM BrdU labeling solution, and reincubated for 16 hours. BrdU incorporation was assessed according to the manufacturer's protocol. In order to ensure that the absorbance obtained was a result of proliferating cells, and not simply from a higher number of viable cells, a 96-well plate containing U251 and SNB-19 cells plated and treated in the same conditions as described for BrdU assay, was done to perform MTT assay, following the method described above. Results are presented as the ratio between BrdU positive cells and MTT positive (viable) cells and represent the mean ± SD of three independent experiments.

### 4.2.7 Chicken Chorioallantoic Membrane (CAM) Assay

CAM assay was performed as previously described[49]. Briefly, fertilized chicken eggs (supplied by Pinto Bar, Portugal) were incubated at 37 °C in a humidified atmosphere, and on day 3 of development, a window was made into the eggshell after puncturing the air chamber, and eggs were sealed with BTK tape and returned to the incubator. On day 9 of development, $2x10^6$ U251 cells, previously exposed to HUCPVCs CM or control media during 4 days, were re-suspended on 10 µL of Matrigel (BD Biosciences), placed on the CAM, and the eggs were tapped and returned to the incubator. At days 11, 13 and 15 of incubation, 100 µL of new CM or control media was added to each respective group. On developmental day 17, tumors were photographed *in ovo* using a stereomicroscope (Olympus S2x16). The chicken embryos were sacrificed at -80°C for 10 minutes. CAMs and tumors were dissected, fixed in 4 % paraformaldehyde at room temperature, and photographed *ex ovo*. The area of the tumors was measured using Cell B software (Olympus), and blood vessels from a selected area containing the tumor were quantified using the Image J software. A total of 38 fertilized chicken eggs were used, 14 in the control group and 24 in the HUCPVCs CM group.

### 4.2.8 Proteomics Analysis

#### 4.2.8.1 Sample preparation

HUCPVCs CM and control media spiked with the same amount of the recombinant protein *mal*E-GFP (to be use as internal standard) were firstly concentrated using a Vivaspin 20 sample concentrator (5 kDa; GE Healthcare) by centrifugation at 3000 *g*. Concentrated CM and control media were precipitated with Trichloroacetic acid (TCA)-Acetone[50]. The washed pellets were ressuspended in 2× Laemmli buffer (BioRad)), aided by ultrasonication and denaturated at 95 °C [51]. After denaturation, samples were alkylated with acrylamide and subjected in gel digestion by using the short-GeLC approach [52]. The entire lanes were sliced into 3 parts, and each part was sliced in small pieces and processed. Gel pieces were destained, dehydrated and re-hydrated in 75 µL of trypsin (0.01 µg/µL solution in 10 mM ammonium bicarbonate) for 15 minutes, on ice. Thirty µL of 10 mM ammonium bicarbonate were then added and in-gel digestion was performed overnight, at room temperature. After digestion, the formed peptides were extracted from the gel pieces by sequential addition of three solutions of acetonitrile (ACN) in 1 % formic acid (FA) (30%, 50%, and 98% of ACN, respectively). All the peptides were dried and subjected to SPE using OMIX tips with C18 stationary phase (Agilent Technologies) as recommended by the manufacture. Eluates were dried and ressuspended with a solution of 2 % ACN and 0.1% FA.

#### 4.2.8.2 SWATH-MS Acquisition

Samples were analyzed on a Triple TOF$^{TM}$ 5600 System (ABSciex®) in two different phases: information-dependent acquisition (IDA) and SWATH acquisition. Peptides were resolved by liquid chromatography (nanoLC Ultra 2D, Eksigent®) on a MicroLC column ChromXP$^{TM}$ C18CL (300 μm ID × 15 cm length, 3 μm particles, 120 Å pore size, Eksigent®) at 5 μL/minutes with a multistep gradient: 0-2 minutes linear gradient from 5 to 10%, 2-45 minutes linear gradient from 10% to 30% and, 45-46 minutes to 35% of ACN in 0.1% FA. Peptides were eluted into the mass spectrometer using an electrospray ionization source (DuoSpray$^{TM}$ Source, ABSciex®) with a 50 μm internal diameter (ID) stainless steel emitter (NewObjective). IDA experiments were performed for each 3 peptide mixtures per samples. The mass spectrometer was set to scanning full spectra (350-1250 m/z) for 250 ms, followed by up to 100 MS/MS scans (100-1500 m/z from a dynamic accumulation time - minimum 30 ms for precursor above the intensity threshold of 1000 - with the purpose of maintaining a cycle time of 3.3 s). Candidate ions with a charge state between +2 and +5 and counts above a minimum threshold of 10 counts per second were isolated for fragmentation and one MS/MS spectra was collected before adding those ions to the exclusion list for 25 seconds (mass spectrometer operated by Analyst® TF 1.7, ABSciex®). Rolling collision was utilized with a collision energy spread of 5.

The 3 peptide mixtures of each sample were combined and concentrated, and a single analysis of each sample was set for quantitative analysis by acquisition in SWATH mode. For SWATH-MS based experiments, the mass spectrometer was operated in a looped product ion mode[53] and the same chromatographic conditions used as in the IDA run described above. The SWATH-MS setup was specifically designed for the samples to be analyzed (Supplementary Table 4.1), in order to adapt the SWATH windows to the complexity of the set of samples. A set of 60 windows of variable width (containing 1 m/z for the window overlap) was conceived covering the precursor mass range of 350-1250 m/z. A 250 ms survey scan (350-1500 m/z) was acquired at the beginning of each cycle and SWATH MS/MS spectra were collected from 100-1500 m/z for 50 ms resulting in a cycle time of 3.25 s from the precursors ranging from 350 to 1250 m/z. The collision energy for each window was determined according to the calculation for a charge +2 ion centered upon the window with variable collision energy spread (CES) according with the window.

A specific library of precursor masses and fragment ions was created by combining all files from the IDA experiments, and used for subsequent SWATH processing. Peptide identification and library generation were performed with Protein Pilot software (v5.1, ABSciex®), using the following parameters: i) search against a database composed by *Homo Sapiens* from SwissProt (release at April 2016), and *malE*-GFP; ii) acrylamide alkylated cysteines as fixed modification; and iii) trypsin as digestion type. An independent false discovery rate (FDR) analysis using the target-decoy approach provided with Protein Pilot software was used to assess the quality of the identifications and positive identifications were considered when identified proteins and peptides reached a 5% local FDR[54, 55]. Data processing was performed using SWATH$^{TM}$ processing plug-in for PeakView$^{TM}$ (v2.0.01, ABSciex®) as described in[52]. After retention time adjustment using the *mal*E-GFP peptides, up to 15 peptides, with up to 5 fragments each, were chosen per protein, and quantitation was attempted for all proteins in the library file that were identified below 5 % local FDR from ProteinPilot$^{TM}$ searches. Peptides' confidence threshold was determined based on a FDR analysis using the target-decoy approach and the peptides that met the 1 % FDR threshold in HUCPVCs sample were retained, and the peak areas of the target fragment ions of those peptides were extracted across the experiments using an extracted-ion chromatogram (XIC) window of 4 minutes and a XIC width of 100 ppm. The levels of the human proteins were estimated by summing all the filtered transitions from all the filtered peptides for a given protein (an adaptation of [56]) normalized to the internal standard (*malE*-GFP).

### 4.2.9 Functional clustering analysis

The identified expressed proteins in HUCPVCs CM were analyzed using the DAVID (Database for Annotation, Visualization and Integrated Discovery) bioinformatics resources version 6.7 (https://david.ncifcrf.gov/)[57, 58]. The list of Uniprot Accession IDs was loaded into the online tool and mapped against reference *Homo sapiens* dataset to extract and summarize functional classification. In DAVID analyses the proteins identified were displayed in Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene ontology (GO), or Reactome pathways.

### 4.2.10 Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad software, Inc.). To assess the statistical differences between groups, unpaired Student's *t*-test analysis was performed. $IC_{50}$ values were calculated by a nonlinear regression (curve Fit) based on sigmoidal dose-response (variable slope), and two-way repeated-measures analysis of variance (ANOVA) test was used to assess statistical differences between conditions. Results are presented as normalized means ± SD, and statistical significance was defined as $p < 0.05$ for a 95% confidence interval.

## 4.3 Results

### 4.3.1 HUCPVCs conditioned media (CM) enhance glioblastoma cell viability, migration and proliferation, and do not affect sensitivity to temozolomide chemotherapy

Taking into consideration the controversial reports on the roles of MSCs on tumor behavior, we started by evaluating how the secretome of HUCPVCs modulates critical hallmark features of GBM cells, particularly viability, proliferation and migration. Using two GBM cell lines, U251 and SNB-19, and CM from HUCPVCs, we evaluated GBM cell viability using two complementary assays: MTT (Figure 4.1A and B) and trypan blue (Figure 4.1C and D). Both U251 and SNB-19 cell lines presented a statistically significant increase in cell viability after exposure to HUCPVCs CM, in all tested time points (shown both by MTT and trypan blue assays; Figure 4.1).

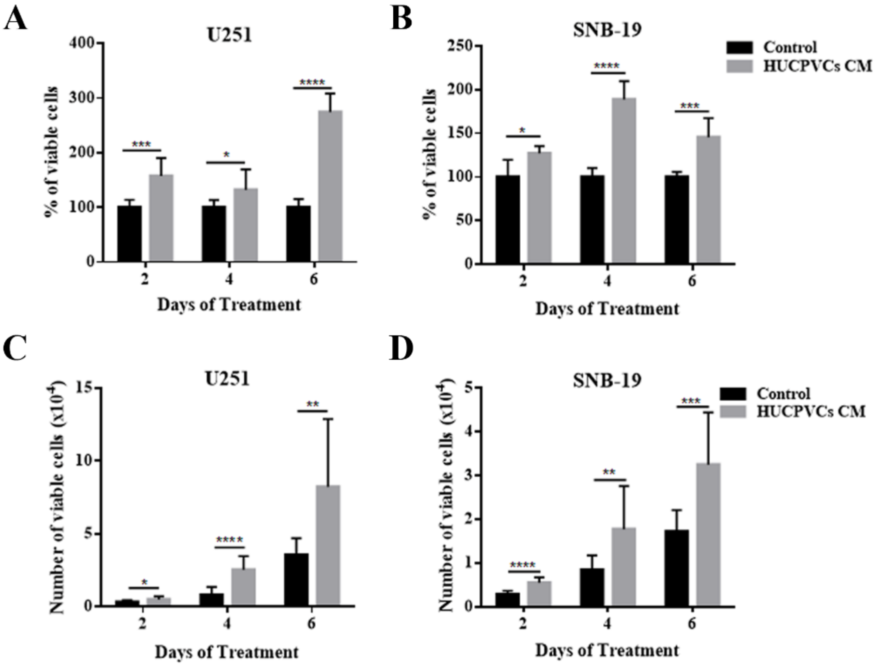

**Figure 4.1: Effect of HUCPVCs conditioned media (CM) on GBM cell viability.** Cell viability was measured by MTT (**A-B**) and trypan blue (**C-D**) assays on U251 (**A** and **C**) and SNB-19 (**B** and **D**) GBM cell lines, after exposure to control media or HUCPVCs CM. HUCPVCs CM led to a statistically significant increase in viability of GBM cells in both assays, in all tested time points. All experiments were done in triplicate, at least in 3 independent experiments. Data is presented as the mean ± SD (*$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$).

GBM cell migration was evaluated by a wound healing assay (Figure 4.2) on U251 (Figure 4.2A and B) and SNB-19 (Figure 4.2C and D) cells exposed to HUCPVCs CM. We found that both GBM cell lines, when exposed to CM, presented a statistically significant higher migration capacity when compared to control/unexposed conditions (Figure 4.2).

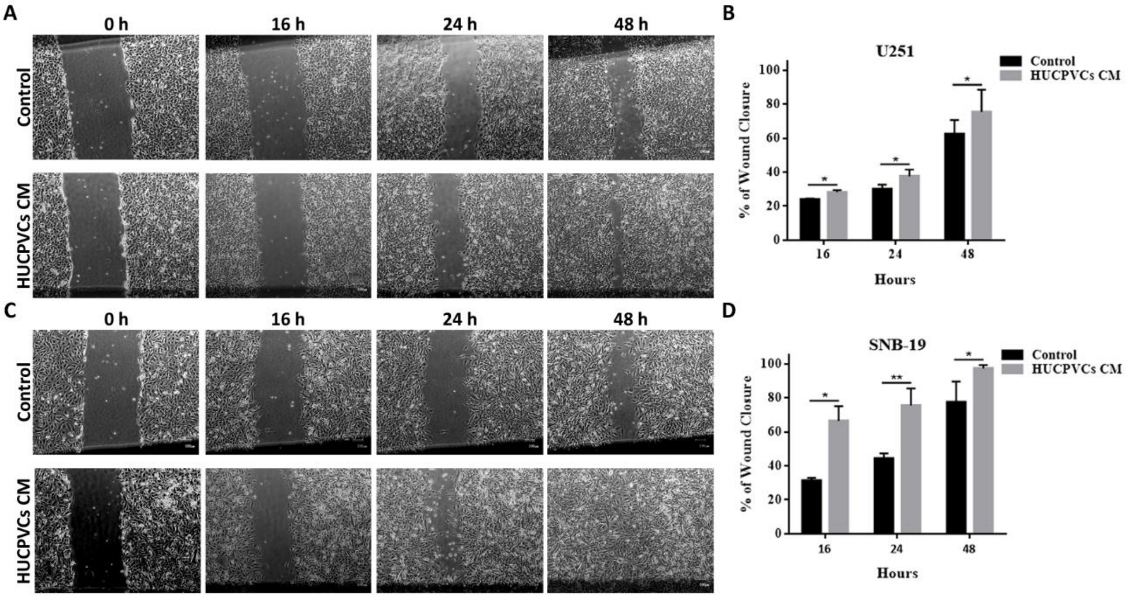

**Figure 4.2: Effect of HUCPVCs conditioned media (CM) on GBM cell migration.** (**A** and **C**) Representative pictures showing the migratory capacity of U251 (**A**) and SNB-19 (**C**) GBM cells exposed to control media or HUCPVCs CM. (**B** and **C**) Quantification of U251 (**B**) and SNB-19 (**D**) cell migration presented as % of wound closure. Treatment with HUCPVCs CM led to a statistically significant increase of GBM cell migration. Data is presented as the mean ± SD of at least 3 independent experiments, each in triplicate (*$p \leq 0.05$ and ** $p \leq 0.01$).

Subsequently, the effect of HUCPVCs CM on GBM cell proliferation was evaluated by the BrdU cell proliferation assay (Figure 4.3A and C). Both U251 (Figure 4.3A) and SNB-19 (Figure 4.3C) GBM cells exposed to HUCPVCs CM showed a statistically significant increase in cell proliferation when compared with control conditions.

The influence of HUCPVCs CM exposure on the response of GBM cells to temozolomide (TMZ)-based chemotherapy was then evaluated (Figure 4.3B and D). The half inhibitory concentration ($IC_{50}$) values after 5 days of TMZ treatment were determined for U251 (Figure 4.3B) and SNB-19 (Figure 4.3D) cells. In contrast to the notorious effects previously observed in cell viability, migration, and proliferation, no significant differences were observed in the sensitivity of U251 and SNB-19 cells to TMZ when exposed to HUCPVCs CM *versus* their respective controls (Figure 4.3B and D).

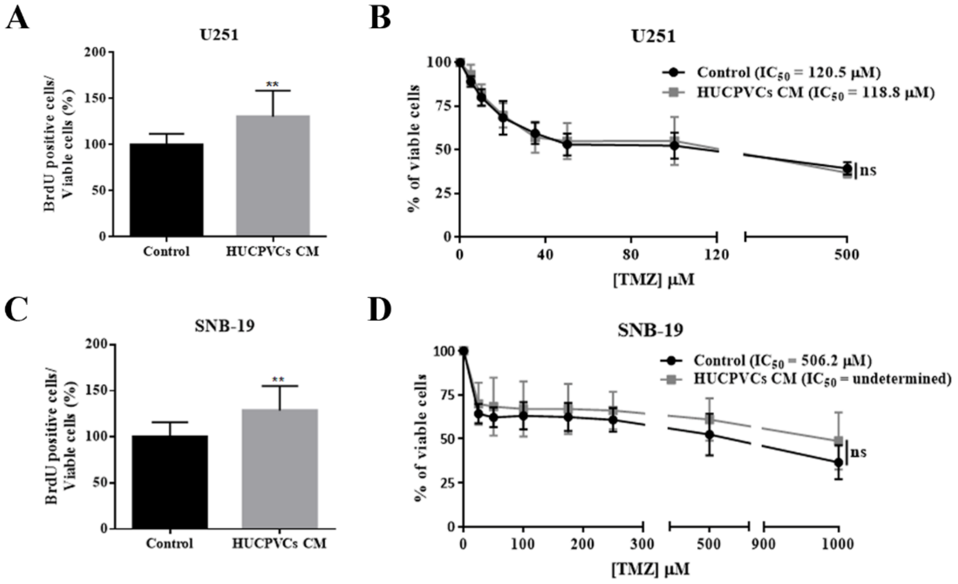

**Figure 4.3: Effect of HUCPVCs conditioned media (CM) on GBM cell proliferation and sensitivity to temozolomide treatment.** (**A** and **C**) Proliferation capacity of U251 (**A**) and SNB-19 (**C**) GBM cells was determined by BrdU assay after treatment with control media or HUCPVCs CM. Exposure to HUCPVCs CM increased the proliferation rate of both GBM cell lines (**$p \leq 0.01$). (**B** and **D**) Determination of the half inhibitory concentration ($IC_{50}$) values of temozolomide (TMZ) treatment in U251 (**B**) and SNB-19 (**D**) cell lines. For both cell lines, no statistically significant differences in the TMZ $IC_{50}$ values were found between cells treated with HUCPVCs CM or control media ($p = 0.6738$ for U251, and $p = 0.3115$ in SNB-19). Results are expressed as the mean ± SD of 3 independent experiments, each in triplicate.

### 4.3.2 HUCPVCs conditioned media (CM) increase *in vivo* tumor growth of U251 GBM cells

In order to complement the *in vitro* studies, the effect of HUCPVCs CM on GBM 3D tumor growth and angiogenesis was then evaluated using the *in vivo* Chick Chorioallantoic Membrane (CAM) assay, which allows efficient tumor formation and vascularization [49]. Concordantly with the *in vitro* data, U251 GBM cells exposed to HUCPVCs CM implanted in the CAM formed statistically significantly larger tumors than those derived from cells exposed to control media ($p = 0.0260$; Figure 4.4A and B). No statistically significant differences were found in vessel density between control- and CM-exposed GBM tumors ($p = 0.0956$; Figure 4.4C and D).

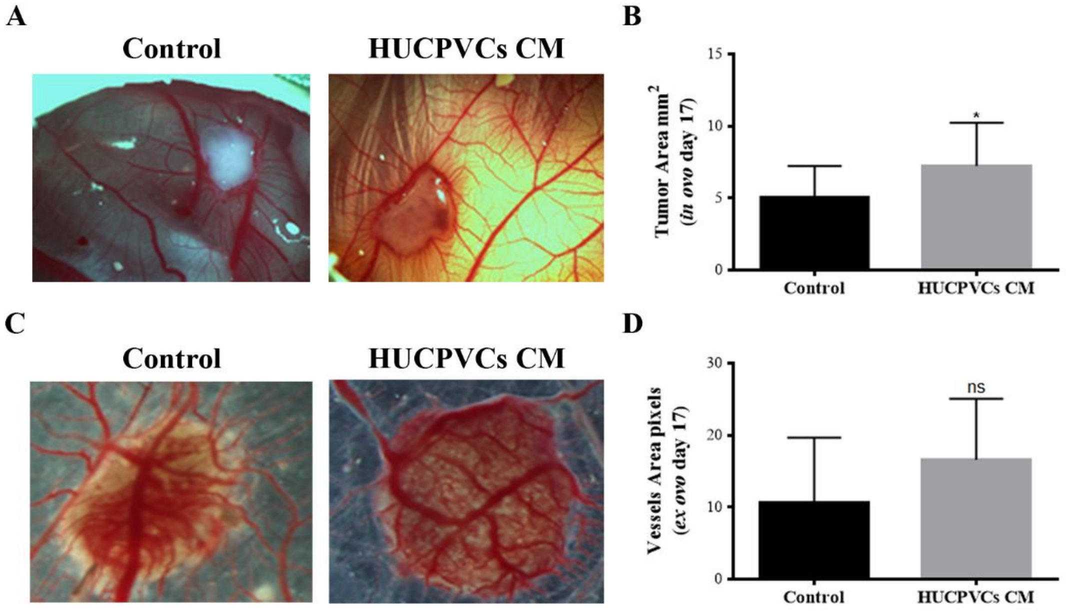

**Figure 4.4**: **Effect of HUCPVCs conditioned media (CM) in U251 GBM cell growth and angiogenesis, *in vivo* CAM model.** Representative pictures of CAM assay after 8 days of tumor growth *in ovo* (**A**) and *ex ovo* (**C**) (16× magnification). (**B**) Tumor growth was measured *in ovo*. A higher tumor area was found in tumors originated from U251 cells exposed to HUCPVCs CM ($p$ = 0.0260). (**D**) Number of blood vessels surrounding tumors. No differences were found in the number of blood vessels between control and cells treated with HUCPVCs CM. Results are expressed as the mean ± SD ($^*p \leq 0.05$).

### 4.3.3 HUCPVCs conditioned media (CM) contain key proteins involved in cell viability, migration, and proliferation, commonly dysregulated in GBM

Considering the broad consistent effects of HUCPVCs CM on the behavior of tumor cells both *in vitro* and *in vivo*, we performed proteomic analyses of HUCPVC CM to identify the protein content of their secretome that may putatively influence GBM behavior. A total of 699 proteins were identified in our proteomic analysis (N = 1; Supplementary Table 4.2).

To better understand the biological functions of these secreted proteins, we employed functional clustering annotation using and integration into Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome analyses (Figure 4.5). Biological processes and cellular components related to extracellular matrix (ECM) organization were the most enriched in HUCPVCs CM (Figure 4.5A and B), whereas actin binding was the most represented molecular function among all identified proteins (Figure 4.5C). Proteins involved in cell cycle, adhesion, motion, survival, migration, and differentiation, which are well known to be key regulators of a variety of physiological processes but also to be dysregulated in cancer cells, were amongst the most abundantly identified (Figure 4.5A). The HUCPVCs secretome was enriched for several pathways by Reactome and KEGG analyses (Figure 4.5D and E), including Wnt, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and pentose phosphate signaling pathways, as well as proteins involved in focal adhesion, ECM-receptor interaction and DNA replication. Globally, these data identify a set of biological processes and pathways that are well-known to be involved in the regulation of physiological *processes, but also to be altered* in cancer, which may partly explain the effects of HUCPVCs CM observed in GBM cell behavior.

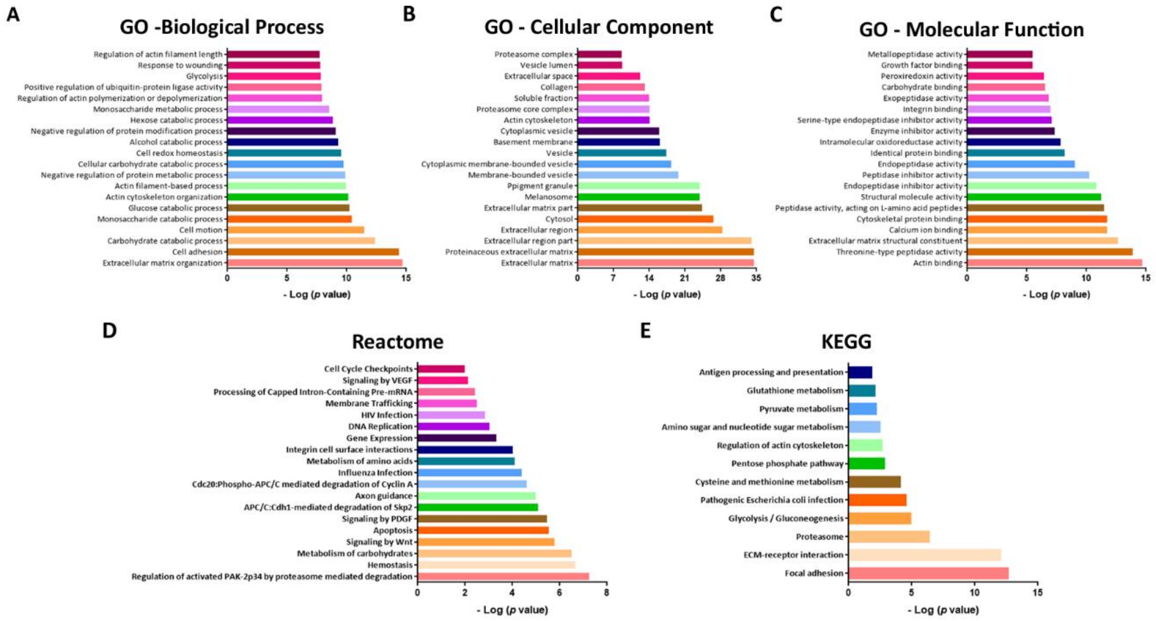

**Figure 4.5**: **Functional analysis of proteins present in HUCPVCs conditioned media (CM). (A-C)** DAVID was used to query the functional annotation of HUCPVCs secretome. The top 20 statistically significant enriched Gene Ontology (GO) terms in Biological Process (**A**), Molecular Component (**B**) and Molecular Function (**C**) are shown. (**D-E**) All statistically significant enriched Reactome (**D**) and KEGG (**E**) pathways are represented. The -log values of *p*-values are displayed.

## 4.4 Discussion

One of the major concerns in stem cell-based therapies is the impact that modified stem cells may have on tumor behavior. MSCs have been proposed as a new therapeutic approach for glioma treatment, because these cells have been found to have tumor chemotactic capabilities and migrate towards tumor sites through the blood-brain-barrier, where continuous bilateral molecular crosstalks occur between stromal and cancer cells[59, 60]. Although some studies suggested that MSCs inhibit tumor growth, others demonstrated that MSCs have a pro-tumoral function by stimulating tumor growth, migration, invasion, and anticancer-drug resistance[3, 12, 13, 61]. The pro-tumoral effects are mediated by secreted molecules and/or via direct cellular interactions[10, 15, 33-35, 62-69]. Therefore, the clinical validity of MSCs as a potential therapeutic approach for glioma is still a matter of debate, deserving further clarification. In this study, we evaluated the influence of HUCPVCs CM on GBM aggressiveness and highlighted proteins from HUCPVCs CM potentially involved in the observed effects.

Our data shows that GBM cells exposed to HUCPVCs CM exhibit increased viability, migration and proliferation *in vitro* (Figures 4.1 - 4.3A and C). Interestingly, the *in vivo* CAM model also showed an increase in tumor growth when GBM cells were exposed to HUCPVCs CM (Figure 4.4). To the best of our knowledge, this is the first study on the influence of HUCPVCs CM in critical hallmark features of GBM.

Previous studies in different tumor types, including gliomas, are in agreement with our results, showing that MSCs may contribute to tumor growth/proliferation[3, 4, 14, 39]. Additionally, it was also demonstrated that factors released by MSCs increased the migration ability of several types of cancer cells, including breast[70], colon[71] and gastric[72] cancers. Regarding gliomas, Onzi and colleagues demonstrated that ASCs CM treatment was able to increase the migration capacity of U87 GBM cells, which is in line with our results[73]. Interestingly, despite the prominent effects of HUCPVCs CM in multiple dimensions of GBM cell biology, the sensitivity of these tumor cells to TMZ chemotherapy was not significantly affected by HUCPVCs CM (Figure 4.3B and D). These results are in agreement with the work of Onzi and colleagues, where they demonstrated that ASCs CM treatment did not alter the response of U87 GBM cells to TMZ[73]. This absence of effect on the response of an anti-tumor drug (doxorubicin) was also recently observed in lung cancer cell lines when exposed to CM from Wharton's jelly derived MSCs by Hendijani and co-workers[16]. Our study is the first to evaluate the influence of HUCPVCs CM on glioma growth and angiogenesis in a CAM assay with formation of 3D microtumors (Figure 4.4).

It is widely accepted that the major mechanism by which MSCs influence cancer pathophysiology is mediated by paracrine events[74-76]. In order to identify which factors secreted by HUCPVCs could be modulating the viability, proliferation, and migration of GBM cells, we performed proteomic analyses of HUCPVCs CM, identifying 699 proteins in the secretome. The functional clustering annotation and integration analyses (Figure 4.5) revealed that HUCPVCs secretome as an enrichment for several pathways (e.g. Wnt, PDGF and VEGF signaling pathways) that have been consistently found dysregulated in cancer, and are known to mediate the phenotypes observed in GBM cells exposed to HUCPVCs CM (namely the increase in proliferation, migration, and invasion), further supporting our experimental findings[77-79]. Similarly, several proteins presented in the secretome of HUCPVCs, such as C-C motif chemokine 2 (CCL2), platelet-derived growth factor C (PDGFC), semaphorin-7A

(SEM7A) (Table 4.1), are known to be important regulators of homeostasis in a variety of physiological conditions, but have also been described to influence tumor cell behavior, as is the case of a classic proto-oncogene.

In conclusion, this work shows that HUCPVCs-secreted molecules increase GBM cell proliferation, migration and viability *in vitro,* accompanied by higher tumor growth *in vivo*. The proteomic characterization identifies several proteins that are putative modulators of these effects in GBM, warranting the need for further studies to understand their mechanisms of action on cancer cells. Our findings also contribute to the understanding of how tumor cells respond to MSCs-released factors, which raises concerns about the safety of their use as clinical tools for the treatment of GBM. Therefore, and taking into account that some studies demonstrated that MSCs can be safely used as drug delivery agents for these purposes[10, 39-41], it is crucial to standardize the methods used in different studies in order to more accurately understand if MSCs are definitely a valid and safe therapeutic approach to tackle cancer. Future studies should have into account several aspects, such as, tissue source and *in vitro* culture conditions of MSCs; type of tumor cells; variability of experimental methodology; and studies using modified MSCs should include unmodified MSCs as control. Importantly, additional studies should be performed using primary GBM cell lines, as well as freshly isolated MSCs, since these cells resembles more the tissue characteristics, and therefore are more physiologically relevant, and are considered closer to the *in vivo* models.

**Table 4.1: Examples of proteins secreted by HUCPVCs that have been described to influence tumor cells' behavior.**

| **Protein** | **Findings in the context of cancer cells** | **References** |
|---|---|---|
| C-C motif chemokine 2 (CCL2) | CCL2 regulates migration and invasion in several cancer types, including gliomas. | 80-82 |
| Actin-related protein 2/3 complex subunit 5 (ARPC5) | ARPC5 contributes to cell migration and invasion in head and neck squamous cell carcinoma. | 83 |
| Translationally-controlled tumor protein (TCTP) | TCTP is overexpressed in glioma tissue and is associated with tumor progression and poor clinical outcome of glioma patients. TCTP promotes glioma cell viability and proliferation, *in vitro*. | 84, 85 |
| Platelet-derived growth factor C (PDGF-C) | PDGF-C plays an important role in glioma vessel maturation and stabilization and in the progression of brain tumors, such as glioblastoma and medulloblastoma; and promotes tumor growth by recruitment of cancer-associated fibroblasts. | 86-88 |
| Alpha-actinin-4 (ACTN4) | ACTN4 enhances the motility and invasion potential of various carcinoma cell lines. | 89 |
| Testican-1 | Testican-1 promotes the proliferation, migration and invasion and inhibits apoptosis in glioma cells. | 90 |
| Neuropilin-2 (NRP-2) | NRP-2 is essential for breast cancer tumor initiation being involved in the formation of focal adhesions and is associated with metastasis and poor prognosis; and promotes the invasion and migration of thyroid cancer cells. | 91-94 |
| Disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) | ADAM10 expression correlates with the grade of malignancy in human glioma; increases the migration capacity of glioma stem cells, and is implicated in U87 cell invasiveness. | 95-97 |
| Transforming growth factor-beta-induced protein (TGFβIp)/ig-h3 | TGFβIp/ig-h3 promotes cell adhesion of human astrocytoma cells, *in vitro*. | 98 |
| Plasminogen activator inhibitor 1 (PAI-1) | PAI-1 is essential in processes related to tumor development, like angiogenesis, adhesion, migration, invasion and metastasis. | 99 |
| Semaphorin-7A (Sema7A) | Sema7A contributes to the increases motility and decreases adhesion necessary for U87 cell invasion. | 95 |
| Periostin | Secreted periostin promotes glioma cell invasion and adhesion. | 100 |

**Note:** The presented proteins were selected from the top 100 most abundant in HUCPVCs CM when compared to control medium.

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## 4.6 Supplementary Information

**Supplementary Table 4.1:** SWATH-MS method.

| | m/z range | Width (Da) | CES |
|---|---|---|---|
| Window 1 | 349.5-364.5 | 15 | 5 |
| Window 2 | 363.5-380.6 | 17.1 | 5 |
| Window 3 | 379.6-395 | 15.4 | 5 |
| Window 4 | 394-408.5 | 14.5 | 5 |
| Window 5 | 407.5-420.7 | 13.2 | 5 |
| Window 6 | 419.7-432.4 | 12.7 | 5 |
| Window 7 | 431.4-442.7 | 11.3 | 5 |
| Window 8 | 441.7-452.2 | 10.5 | 5 |
| Window 9 | 451.2-461.6 | 10.4 | 5 |
| Window 10 | 460.6-470.2 | 9.6 | 5 |
| Window 11 | 469.2-478.7 | 9.5 | 5 |
| Window 12 | 477.7-487.3 | 9.6 | 5 |
| Window 13 | 486.3-494.9 | 8.6 | 5 |
| Window 14 | 493.9-503 | 9.1 | 5 |
| Window 15 | 502-510.7 | 8.7 | 5 |
| Window 16 | 509.7-518.8 | 9.1 | 5 |
| Window 17 | 517.8-526.4 | 8.6 | 5 |
| Window 18 | 525.4-533.6 | 8.2 | 5 |
| Window 19 | 532.6-541.3 | 8.7 | 5 |
| Window 20 | 540.3-549 | 8.7 | 5 |
| Window 21 | 548-556.2 | 8.2 | 5 |
| Window 22 | 555.2-564.3 | 9.1 | 5 |
| Window 23 | 563.3-571.9 | 8.6 | 5 |
| Window 24 | 570.9-579.1 | 8.2 | 5 |
| Window 25 | 578.1-586.8 | 8.7 | 5 |
| Window 26 | 585.8-594.4 | 8.6 | 5 |
| Window 27 | 593.4-602.5 | 9.1 | 5 |
| Window 28 | 601.5-610.2 | 8.7 | 5 |
| Window 29 | 609.2-618.3 | 9.1 | 5 |
| Window 30 | 617.3-625.9 | 8.6 | 5 |
| Window 31 | 624.9-634 | 9.1 | 5 |

| | | | |
|---|---|---|---|
| Window 32 | 633-642.6 | 9.6 | 5 |
| Window 33 | 641.6-651.1 | 9.5 | 5 |
| Window 34 | 650.1-660.1 | 10 | 5 |
| Window 35 | 659.1-668.7 | 9.6 | 5 |
| Window 36 | 667.7-678.1 | 10.4 | 5 |
| Window 37 | 677.1-687.1 | 10 | 5 |
| Window 38 | 686.1-697 | 10.9 | 5 |
| Window 39 | 696-706.9 | 10.9 | 5 |
| Window 40 | 705.9-716.8 | 10.9 | 5 |
| Window 41 | 715.8-727.2 | 11.4 | 5 |
| Window 42 | 726.2-738 | 11.8 | 5 |
| Window 43 | 737-748.8 | 11.8 | 5 |
| Window 44 | 747.8-760.5 | 12.7 | 5 |
| Window 45 | 759.5-772.6 | 13.1 | 5 |
| Window 46 | 771.6-785.7 | 14.1 | 5 |
| Window 47 | 784.7-799.2 | 14.5 | 5 |
| Window 48 | 798.2-813.6 | 15.4 | 8 |
| Window 49 | 812.6-829.3 | 16.7 | 8 |
| Window 50 | 828.3-845.1 | 16.8 | 8 |
| Window 51 | 844.1-861.3 | 17.2 | 8 |
| Window 52 | 860.3-878.4 | 18.1 | 8 |
| Window 53 | 877.4-897.7 | 20.3 | 8 |
| Window 54 | 896.7-920.2 | 23.5 | 8 |
| Window 55 | 919.2-949.9 | 30.7 | 8 |
| Window 56 | 948.9-984.1 | 35.2 | 8 |
| Window 57 | 983.1-1020.1 | 37 | 10 |
| Window 58 | 1019.1-1064.2 | 45.1 | 10 |
| Window 59 | 1063.2-1135.3 | 72.1 | 10 |
| Window 60 | 1134.3-1249.6 | 115.3 | 10 |

**Supplementary Table 4.2**. Proteins identified in the secretome of HUCPVCs.

| **No. Identified Peptides** | **No. Quantified Peptides** | **Uniprot Accesion Name** | **Protein Name** | **Relative Protein Levels (normalized to internal standard)** | | **Ratio HUCPVCs/Ctrl** |
|---|---|---|---|---|---|---|
| | | | | **Control** | **HUCPVCs** | |
| 4 | 1 | CCL2_Human | C-C motif chemokine 2 | 0.000 | 0.036 | 709.1 |
| 1 | 1 | MOXD1_Human | DBH-like monooxygenase protein 1 | 0.000 | 0.009 | 221.9 |
| 4 | 1 | ARPC5_Human | Actin-related protein 2/3 complex subunit 5 | 0.000 | 0.008 | 154.0 |
| 1 | 1 | PALLD_Human | Palladin | 0.000 | 0.004 | 104.6 |
| 8 | 5 | TCTP_Human | Translationally-controlled tumor protein | 0.000 | 0.045 | 92.2 |
| 1 | 1 | LYOX_Human | Protein-lysine 6-oxidase | 0.000 | 0.002 | 87.2 |
| 54 | 15 | MMP2_Human | 72 kDa type IV collagenase | 0.017 | 1.421 | 84.3 |
| 1 | 1 | PDGFC_Human | Platelet-derived growth factor C | 0.000 | 0.002 | 77.6 |
| 61 | 15 | PXDN_Human | Peroxidasin homolog | 0.003 | 0.219 | 72.4 |
| 32 | 14 | PDIA3_Human | Protein disulfide-isomerase A3 | 0.004 | 0.266 | 70.4 |
| 59 | 14 | ACTN4_Human | Alpha-actinin-4 {ECO:0000305} | 0.003 | 0.220 | 67.8 |
| 8 | 6 | TICN1_Human | Testican-1 | 0.001 | 0.045 | 67.4 |
| 2 | 1 | ADHX_Human | Alcohol dehydrogenase class-3 | 0.000 | 0.006 | 66.5 |
| 4 | 2 | PTPA_Human | Serine/threonine-protein phosphatase 2A activator | 0.000 | 0.010 | 63.4 |
| 39 | 14 | QSOX1_Human | Sulfhydryl oxidase 1 | 0.007 | 0.412 | 63.1 |
| 1 | 1 | STIP1_Human | Stress-induced-phosphoprotein 1 | 0.000 | 0.002 | 62.6 |
| 33 | 14 | C1R_Human | Complement C1r subcomponent | 0.005 | 0.294 | 62.4 |
| 26 | 11 | CO5A1_Human | Collagen alpha-1(V) chain | 0.002 | 0.139 | 62.2 |
| 2 | 1 | EPCR_Human | Endothelial protein C receptor | 0.000 | 0.007 | 61.8 |
| 18 | 13 | PGS1_Human | Biglycan | 0.007 | 0.422 | 60.6 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 13 | 8 | TXND5_Human | Thioredoxin domain-containing protein 5 | 0.001 | 0.064 | 60.3 |
| 1 | 1 | SEC13_Human | Protein SEC13 homolog | 0.000 | 0.002 | 60.3 |
| 1 | 1 | NIF3L_Human | NIF3-like protein 1 {ECO:0000305\|PubMed:11124544} | 0.000 | 0.003 | 60.0 |
| 42 | 15 | NID2_Human | Nidogen-2 | 0.003 | 0.182 | 60.0 |
| 3 | 2 | BTD_Human | Biotinidase | 0.000 | 0.014 | 59.2 |
| 2 | 2 | CO8A1_Human | Collagen alpha-1(VIII) chain | 0.000 | 0.008 | 57.9 |
| 21 | 15 | PTX3_Human | Pentraxin-related protein PTX3 | 0.024 | 1.346 | 56.8 |
| 33 | 14 | NRP2_Human | Neuropilin-2 | 0.002 | 0.128 | 56.1 |
| 37 | 15 | CSTN1_Human | Calsyntenin-1 | 0.012 | 0.660 | 55.5 |
| 22 | 14 | GDN_Human | Glia-derived nexin | 0.006 | 0.312 | 55.5 |
| 95 | 14 | FLNA_Human | Filamin-A | 0.005 | 0.299 | 55.2 |
| 3 | 2 | NUCB2_Human | Nucleobindin-2 | 0.000 | 0.007 | 54.3 |
| 6 | 3 | ADA10_Human | Disintegrin and metalloproteinase domain-containing protein 10 | 0.000 | 0.016 | 54.0 |
| 1 | 1 | STRAP_Human | Serine-threonine kinase receptor-associated protein | 0.000 | 0.001 | 53.9 |
| 2 | 1 | S10A6_Human | Protein S100-A6 | 0.000 | 0.005 | 53.8 |
| 13 | 11 | PNPH_Human | Purine nucleoside phosphorylase | 0.002 | 0.088 | 52.4 |
| 37 | 15 | ACTN1_Human | Alpha-actinin-1 | 0.006 | 0.319 | 52.3 |
| 33 | 15 | TSP2_Human | Thrombospondin-2 | 0.006 | 0.322 | 52.0 |
| 8 | 3 | STC2_Human | Stanniocalcin-2 | 0.002 | 0.102 | 50.1 |
| 2 | 1 | FBLN5_Human | Fibulin-5 | 0.000 | 0.008 | 49.3 |
| 30 | 15 | PLST_Human | Plastin-3 | 0.005 | 0.245 | 48.6 |
| 47 | 16 | BGH3_Human | Transforming growth factor-beta-induced protein ig-h3 | 0.044 | 2.137 | 48.3 |
| 8 | 7 | CTHR1_Human | Collagen triple helix repeat-containing protein 1 | 0.003 | 0.151 | 48.3 |
| 108 | 15 | CO6A3_Human | Collagen alpha-3(VI) chain | 0.004 | 0.214 | 47.9 |
| 4 | 2 | IF4A2_Human | Eukaryotic initiation factor 4A-II | 0.000 | 0.010 | 47.8 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 29 | 13 | CO5A2_Human | Collagen alpha-2(V) chain | 0.003 | 0.126 | 47.6 |
| 2 | 1 | ARP2_Human | Actin-related protein 2 | 0.000 | 0.006 | 47.4 |
| 11 | 5 | PEPD_Human | Xaa-Pro dipeptidase | 0.001 | 0.030 | 47.3 |
| 4 | 3 | RBMX_Human | RNA-binding motif protein, X chromosome | 0.000 | 0.018 | 47.2 |
| 50 | 15 | TSP1_Human | Thrombospondin-1 | 0.012 | 0.576 | 47.1 |
| 58 | 15 | CO6A1_Human | Collagen alpha-1(VI) chain | 0.035 | 1.626 | 46.5 |
| 37 | 15 | PAI1_Human | Plasminogen activator inhibitor 1 | 0.060 | 2.769 | 46.1 |
| 8 | 3 | PSB4_Human | Proteasome subunit beta type-4 | 0.001 | 0.031 | 46.1 |
| 20 | 14 | CATB_Human | Cathepsin B | 0.007 | 0.306 | 45.8 |
| 31 | 15 | MANBA_Human | Beta-mannosidase | 0.004 | 0.193 | 45.7 |
| 40 | 15 | MMP1_Human | Interstitial collagenase | 0.037 | 1.683 | 45.7 |
| 37 | 15 | CO4A2_Human | Collagen alpha-2(IV) chain | 0.007 | 0.325 | 45.7 |
| 100 | 15 | FBN1_Human | Fibrillin-1 | 0.009 | 0.392 | 45.5 |
| 29 | 15 | SEM7A_Human | Semaphorin-7A | 0.005 | 0.209 | 44.8 |
| 22 | 14 | FBLN1_Human | Fibulin-1 | 0.003 | 0.143 | 44.6 |
| 1 | 1 | MINP1_Human | Multiple inositol polyphosphate phosphatase 1 | 0.000 | 0.006 | 44.4 |
| 44 | 14 | MOES_Human | Moesin | 0.008 | 0.356 | 44.1 |
| 3 | 1 | CALD1_Human | Caldesmon | 0.000 | 0.003 | 43.8 |
| 96 | 14 | CO3_Human | Complement C3 | 0.008 | 0.367 | 43.7 |
| 4 | 2 | CPPED_Human | Serine/threonine-protein phosphatase CPPED1 | 0.000 | 0.005 | 43.5 |
| 20 | 12 | PGS2_Human | Decorin | 0.004 | 0.186 | 43.4 |
| 2 | 1 | ENOPH_Human | Enolase-phosphatase E1 {ECO:0000255\|HAMAP-Rule:MF_03117} | 0.000 | 0.004 | 42.8 |
| 1 | 1 | SC23A_Human | Protein transport protein Sec23A | 0.000 | 0.002 | 42.8 |
| 9 | 8 | VASN_Human | Vasorin | 0.002 | 0.090 | 42.6 |
| 2 | 1 | BACH_Human | Cytosolic acyl coenzyme A thioester hydrolase | 0.000 | 0.004 | 42.4 |

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|---|---|---|---|---|---|---|
| 23 | 15 | ECM1_Human | Extracellular matrix protein 1 | 0.007 | 0.307 | 42.3 |
| 142 | 15 | FINC_Human | Fibronectin | 0.045 | 1.887 | 42.3 |
| 2 | 2 | PGFRL_Human | Platelet-derived growth factor receptor-like protein | 0.000 | 0.003 | 42.0 |
| 2 | 1 | OTUB1_Human | Ubiquitin thioesterase OTUB1 | 0.000 | 0.003 | 41.9 |
| 87 | 16 | CO1A2_Human | Collagen alpha-2(I) chain | 0.036 | 1.513 | 41.8 |
| 7 | 2 | KAD2_Human | Adenylate kinase 2, mitochondrial {ECO:0000255\|HAMAP-Rule:MF_03168} | 0.000 | 0.009 | 41.8 |
| 3 | 3 | EXTL2_Human | Exostosin-like 2 | 0.000 | 0.009 | 41.6 |
| 39 | 12 | LAMB1_Human | Laminin subunit beta-1 | 0.003 | 0.111 | 41.6 |
| 11 | 5 | TIMP1_Human | Metalloproteinase inhibitor 1 | 0.007 | 0.276 | 41.5 |
| 7 | 6 | LMAN2_Human | Vesicular integral-membrane protein VIP36 | 0.001 | 0.024 | 41.3 |
| 9 | 2 | PRDX5_Human | Peroxiredoxin-5, mitochondrial | 0.000 | 0.013 | 41.2 |
| 4 | 2 | DX39B_Human | Spliceosome RNA helicase DDX39B | 0.000 | 0.008 | 41.1 |
| 17 | 14 | OLFL3_Human | Olfactomedin-like protein 3 | 0.007 | 0.278 | 41.1 |
| 46 | 15 | LTBP1_Human | Latent-transforming growth factor beta-binding protein 1 | 0.003 | 0.127 | 40.9 |
| 12 | 10 | GAS6_Human | Growth arrest-specific protein 6 | 0.001 | 0.060 | 40.9 |
| 3 | 2 | DOPD_Human | D-dopachrome decarboxylase | 0.000 | 0.010 | 40.9 |
| 23 | 12 | LOXL2_Human | Lysyl oxidase homolog 2 | 0.002 | 0.101 | 40.5 |
| 2 | 1 | HMCN1_Human | Hemicentin-1 | 0.000 | 0.002 | 40.4 |
| 29 | 14 | POSTN_Human | Periostin | 0.006 | 0.237 | 40.1 |
| 6 | 3 | NPC2_Human | Epididymal secretory protein E1 | 0.000 | 0.013 | 40.1 |
| 20 | 15 | SPRC_Human | SPARC | 0.027 | 1.097 | 40.0 |
| 3 | 1 | UBC9_Human | SUMO-conjugating enzyme UBC9 | 0.000 | 0.003 | 40.0 |
| 39 | 15 | CFAH_Human | Complement factor H | 0.003 | 0.116 | 39.8 |

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|---|---|---|---|---|---|---|
| 4 | 3 | FABP5_Human | Fatty acid-binding protein, epidermal | 0.000 | 0.018 | 39.7 |
| 35 | 13 | C1S_Human | Complement C1s subcomponent | 0.008 | 0.317 | 39.4 |
| 53 | 14 | CO3A1_Human | Collagen alpha-1(III) chain | 0.015 | 0.589 | 39.4 |
| 2 | 1 | CD81_Human | CD81 antigen | 0.000 | 0.003 | 39.2 |
| 29 | 12 | MA2A1_Human | Alpha-mannosidase 2 | 0.002 | 0.075 | 39.1 |
| 2 | 1 | SGT1_Human | Protein SGT1 homolog {ECO:0000250\|UniProtKB:Q08446} | 0.000 | 0.002 | 39.0 |
| 21 | 15 | LG3BP_Human | Galectin-3-binding protein | 0.007 | 0.268 | 38.5 |
| 10 | 8 | DSG2_Human | Desmoglein-2 | 0.001 | 0.044 | 38.0 |
| 12 | 8 | CO4A1_Human | Collagen alpha-1(IV) chain | 0.002 | 0.064 | 38.0 |
| 4 | 2 | NPTX1_Human | Neuronal pentraxin-1 | 0.000 | 0.007 | 38.0 |
| 4 | 3 | SRPX_Human | Sushi repeat-containing protein SRPX | 0.000 | 0.016 | 37.9 |
| 23 | 12 | GRP78_Human | 78 kDa glucose-regulated protein | 0.005 | 0.174 | 37.7 |
| 1 | 1 | SUMO4_Human | Small ubiquitin-related modifier 4 | 0.000 | 0.002 | 37.7 |
| 15 | 10 | LUM_Human | Lumican | 0.009 | 0.351 | 37.5 |
| 4 | 2 | HNRPD_Human | Heterogeneous nuclear ribonucleoprotein D0 | 0.000 | 0.014 | 37.5 |
| 3 | 3 | PA2G4_Human | Proliferation-associated protein 2G4 | 0.000 | 0.011 | 37.3 |
| 18 | 15 | PLOD2_Human | Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 | 0.003 | 0.102 | 37.2 |
| 19 | 15 | GALT2_Human | Polypeptide N-acetylgalactosaminyltransferase 2 | 0.003 | 0.102 | 37.2 |
| 14 | 11 | HTRA1_Human | Serine protease HTRA1 | 0.002 | 0.076 | 37.1 |
| 14 | 9 | UCHL1_Human | Ubiquitin carboxyl-terminal hydrolase isozyme L1 | 0.004 | 0.156 | 37.1 |
| 5 | 4 | COPA_Human | Coatomer subunit alpha | 0.000 | 0.014 | 36.9 |

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| 36 | 15 | CO6A2_Human | Collagen alpha-2(VI) chain | 0.013 | 0.487 | 36.7 |
| 3 | 3 | NDKA_Human | Nucleoside diphosphate kinase A | 0.001 | 0.021 | 36.5 |
| 3 | 1 | SRP09_Human | Signal recognition particle 9 kDa protein | 0.000 | 0.002 | 36.4 |
| 34 | 15 | NID1_Human | Nidogen-1 | 0.003 | 0.121 | 36.4 |
| 3 | 2 | OAF_Human | Out at first protein homolog | 0.000 | 0.010 | 36.4 |
| 25 | 12 | PDIA1_Human | Protein disulfide-isomerase | 0.004 | 0.153 | 36.1 |
| 14 | 3 | DLDH_Human | Dihydrolipoyl dehydrogenase, mitochondrial | 0.000 | 0.014 | 36.0 |
| 20 | 13 | CALR_Human | Calreticulin | 0.004 | 0.162 | 36.0 |
| 22 | 13 | PCOC1_Human | Procollagen C-endopeptidase enhancer 1 | 0.006 | 0.206 | 35.8 |
| 10 | 7 | EF1A3_Human | Putative elongation factor 1-alpha-like 3 | 0.002 | 0.088 | 35.7 |
| 42 | 15 | LAMC1_Human | Laminin subunit gamma-1 | 0.004 | 0.151 | 35.7 |
| 10 | 7 | CD248_Human | Endosialin | 0.002 | 0.059 | 35.7 |
| 6 | 3 | NUDC_Human | Nuclear migration protein nudC | 0.000 | 0.009 | 35.7 |
| 16 | 11 | SERPH_Human | Serpin H1 | 0.002 | 0.079 | 35.5 |
| 22 | 12 | FSCN1_Human | Fascin | 0.003 | 0.114 | 35.3 |
| 8 | 6 | BMP1_Human | Bone morphogenetic protein 1 | 0.001 | 0.037 | 35.3 |
| 6 | 4 | PSA3_Human | Proteasome subunit alpha type-3 | 0.001 | 0.028 | 34.8 |
| 15 | 10 | MA1A1_Human | Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA | 0.002 | 0.085 | 34.8 |
| 8 | 7 | GGH_Human | Gamma-glutamyl hydrolase | 0.002 | 0.055 | 34.7 |
| 18 | 14 | IBP7_Human | Insulin-like growth factor-binding protein 7 | 0.026 | 0.890 | 34.7 |
| 11 | 6 | PTK7_Human | Inactive tyrosine-protein kinase 7 | 0.001 | 0.036 | 34.7 |
| 19 | 14 | CBPA4_Human | Carboxypeptidase A4 | 0.006 | 0.217 | 34.6 |

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| 3 | 1 | RAB1A_Human | Ras-related protein Rab-1A | 0.000 | 0.006 | 34.6 |
| 3 | 2 | COGA1_Human | Collagen alpha-1(XVI) chain | 0.000 | 0.007 | 34.6 |
| 6 | 4 | AP2B1_Human | AP-2 complex subunit beta | 0.000 | 0.010 | 34.5 |
| 2 | 2 | MFAP2_Human | Microfibrillar-associated protein 2 | 0.000 | 0.008 | 34.4 |
| 33 | 15 | ENOA_Human | Alpha-enolase | 0.022 | 0.743 | 34.3 |
| 19 | 14 | MDHM_Human | Malate dehydrogenase, mitochondrial | 0.006 | 0.218 | 34.2 |
| 16 | 7 | GDIA_Human | Rab GDP dissociation inhibitor alpha | 0.002 | 0.057 | 34.2 |
| 32 | 14 | HSP7C_Human | Heat shock cognate 71 kDa protein | 0.007 | 0.243 | 34.2 |
| 104 | 14 | COCA1_Human | Collagen alpha-1(XII) chain | 0.009 | 0.319 | 33.9 |
| 2 | 1 | RUXE_Human | Small nuclear ribonucleoprotein E | 0.000 | 0.003 | 33.8 |
| 4 | 2 | FBLN4_Human | EGF-containing fibulin-like extracellular matrix protein 2 | 0.000 | 0.007 | 33.7 |
| 4 | 3 | RAN_Human | GTP-binding nuclear protein Ran | 0.001 | 0.026 | 33.7 |
| 7 | 5 | B4GT1_Human | Beta-1,4-galactosyltransferase 1 | 0.001 | 0.044 | 33.6 |
| 2 | 1 | PLD3_Human | Phospholipase D3 | 0.000 | 0.002 | 33.6 |
| 39 | 12 | LTBP2_Human | Latent-transforming growth factor beta-binding protein 2 | 0.004 | 0.136 | 33.5 |
| 4 | 2 | FPPS_Human | Farnesyl pyrophosphate synthase | 0.000 | 0.008 | 33.4 |
| 29 | 11 | LAMA4_Human | Laminin subunit alpha-4 | 0.002 | 0.067 | 33.3 |
| 4 | 2 | PPIC_Human | Peptidyl-prolyl cis-trans isomerase C | 0.001 | 0.017 | 33.3 |
| 3 | 1 | MIF_Human | Macrophage migration inhibitory factor | 0.001 | 0.033 | 33.3 |
| 23 | 11 | EMIL1_Human | EMILIN-1 | 0.002 | 0.065 | 33.0 |
| 3 | 1 | FCL_Human | GDP-L-fucose synthase | 0.000 | 0.003 | 32.8 |
| 5 | 2 | SERB_Human | Phosphoserine phosphatase | 0.000 | 0.008 | 32.6 |

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| 18 | 11 | 1433E_Human | 14-3-3 protein epsilon | 0.005 | 0.165 | 32.4 |
| 23 | 14 | CH3L1_Human | Chitinase-3-like protein 1 | 0.029 | 0.938 | 32.4 |
| 16 | 15 | TPA_Human | Tissue-type plasminogen activator | 0.003 | 0.106 | 32.4 |
| 4 | 4 | MYDGF_Human | Myeloid-derived growth factor {ECO:0000303\|PubMed:25581518, ECO:0000312\|HGNC:HGNC:16948} | 0.002 | 0.058 | 32.1 |
| 3 | 2 | RBBP4_Human | Histone-binding protein RBBP4 | 0.000 | 0.011 | 32.1 |
| 18 | 13 | LKHA4_Human | Leukotriene A-4 hydrolase | 0.002 | 0.049 | 32.1 |
| 1 | 1 | RS10_Human | 40S ribosomal protein S10 | 0.000 | 0.001 | 32.1 |
| 4 | 3 | TSP3_Human | Thrombospondin-3 | 0.000 | 0.007 | 31.8 |
| 3 | 2 | SGCE_Human | Epsilon-sarcoglycan | 0.000 | 0.004 | 31.8 |
| 14 | 13 | IDHC_Human | Isocitrate dehydrogenase [NADP] cytoplasmic | 0.002 | 0.077 | 31.7 |
| 6 | 3 | RL10A_Human | 60S ribosomal protein L10a | 0.001 | 0.031 | 31.7 |
| 41 | 15 | KPYM_Human | Pyruvate kinase PKM | 0.019 | 0.594 | 31.6 |
| 5 | 5 | HEXA_Human | Beta-hexosaminidase subunit alpha | 0.001 | 0.022 | 31.6 |
| 3 | 3 | 4F2_Human | 4F2 cell-surface antigen heavy chain | 0.000 | 0.011 | 31.6 |
| 7 | 5 | CATZ_Human | Cathepsin Z | 0.001 | 0.037 | 31.6 |
| 26 | 13 | TKT_Human | Transketolase | 0.009 | 0.291 | 31.4 |
| 25 | 12 | PGK1_Human | Phosphoglycerate kinase 1 | 0.006 | 0.179 | 31.4 |
| 27 | 12 | GDIB_Human | Rab GDP dissociation inhibitor beta | 0.004 | 0.123 | 31.2 |
| 1 | 1 | CALX_Human | Calnexin | 0.000 | 0.002 | 31.2 |
| 4 | 1 | HGF_Human | Hepatocyte growth factor | 0.000 | 0.003 | 31.0 |
| 2 | 2 | SC22B_Human | Vesicle-trafficking protein SEC22b | 0.000 | 0.003 | 30.9 |
| 6 | 4 | GLGB_Human | 1,4-alpha-glucan-branching enzyme | 0.001 | 0.017 | 30.9 |
| 1 | 1 | LTOR3_Human | Ragulator complex protein LAMTOR3 | 0.000 | 0.002 | 30.8 |

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| 12 | 8 | HSP74_Human | Heat shock 70 kDa protein 4 | 0.001 | 0.038 | 30.8 |
| 11 | 8 | ROA1_Human | Heterogeneous nuclear ribonucleoprotein A1 | 0.004 | 0.110 | 30.5 |
| 37 | 13 | PAPP1_Human | Pappalysin-1 | 0.003 | 0.101 | 30.4 |
| 5 | 5 | RSU1_Human | Ras suppressor protein 1 | 0.001 | 0.017 | 30.4 |
| 21 | 12 | CD109_Human | CD109 antigen | 0.002 | 0.049 | 30.4 |
| 25 | 15 | G6PI_Human | Glucose-6-phosphate isomerase | 0.007 | 0.199 | 30.3 |
| 17 | 13 | CSPG2_Human | Versican core protein | 0.004 | 0.111 | 30.3 |
| 22 | 12 | EXT2_Human | Exostosin-2 | 0.003 | 0.077 | 30.2 |
| 14 | 8 | GSHB_Human | Glutathione synthetase | 0.001 | 0.035 | 30.2 |
| 88 | 15 | CO1A1_Human | Collagen alpha-1(I) chain | 0.071 | 2.125 | 30.1 |
| 12 | 7 | DPYL3_Human | Dihydropyrimidinase-related protein 3 | 0.001 | 0.037 | 29.9 |
| 13 | 9 | HEXB_Human | Beta-hexosaminidase subunit beta | 0.002 | 0.047 | 29.9 |
| 8 | 7 | PDIA6_Human | Protein disulfide-isomerase A6 | 0.001 | 0.033 | 29.8 |
| 27 | 15 | VINC_Human | Vinculin | 0.004 | 0.116 | 29.7 |
| 4 | 2 | FRIL_Human | Ferritin light chain | 0.000 | 0.010 | 29.7 |
| 3 | 2 | ELAV1_Human | ELAV-like protein 1 | 0.000 | 0.005 | 29.6 |
| 6 | 4 | CAPZB_Human | F-actin-capping protein subunit beta | 0.000 | 0.014 | 29.5 |
| 14 | 10 | PEDF_Human | Pigment epithelium-derived factor | 0.003 | 0.085 | 29.4 |
| 4 | 2 | PSME1_Human | Proteasome activator complex subunit 1 | 0.000 | 0.007 | 29.4 |
| 2 | 2 | GLRX3_Human | Glutaredoxin-3 | 0.000 | 0.008 | 29.4 |
| 27 | 15 | ALDOA_Human | Fructose-bisphosphate aldolase A | 0.012 | 0.345 | 29.4 |
| 6 | 2 | LGUL_Human | Lactoylglutathione lyase | 0.000 | 0.013 | 29.3 |
| 6 | 5 | GANAB_Human | Neutral alpha-glucosidase AB | 0.001 | 0.018 | 29.3 |
| 53 | 12 | FLNB_Human | Filamin-B | 0.002 | 0.070 | 29.1 |
| 7 | 4 | TFR1_Human | Transferrin receptor protein 1 | 0.000 | 0.009 | 29.1 |

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|---|---|---|---|---|---|---|
| 19 | 13 | TERA_Human | Transitional endoplasmic reticulum ATPase | 0.002 | 0.049 | 29.1 |
| 9 | 2 | PTBP1_Human | Polypyrimidine tract-binding protein 1 | 0.000 | 0.010 | 29.1 |
| 22 | 13 | SPON2_Human | Spondin-2 | 0.035 | 1.002 | 29.0 |
| 7 | 3 | IMB1_Human | Importin subunit beta-1 | 0.000 | 0.008 | 28.8 |
| 13 | 11 | GSTP1_Human | Glutathione S-transferase P | 0.007 | 0.197 | 28.7 |
| 18 | 8 | AGRIN_Human | Agrin | 0.001 | 0.033 | 28.6 |
| 19 | 13 | LDHB_Human | L-lactate dehydrogenase B chain | 0.012 | 0.340 | 28.6 |
| 2 | 2 | LIS1_Human | Platelet-activating factor acetylhydrolase IB subunit alpha {ECO:0000255\|HAMAP-Rule:MF_03141} | 0.000 | 0.008 | 28.5 |
| 1 | 1 | PA1B3_Human | Platelet-activating factor acetylhydrolase IB subunit gamma | 0.000 | 0.002 | 28.5 |
| 6 | 4 | ENPL_Human | Endoplasmin | 0.001 | 0.019 | 28.2 |
| 11 | 10 | NUCB1_Human | Nucleobindin-1 | 0.002 | 0.056 | 28.2 |
| 2 | 2 | ADA19_Human | Disintegrin and metalloproteinase domain-containing protein 19 | 0.000 | 0.012 | 28.2 |
| 25 | 15 | TAGL_Human | Transgelin | 0.026 | 0.717 | 28.1 |
| 1 | 1 | FUCO_Human | Tissue alpha-L-fucosidase | 0.000 | 0.004 | 28.0 |
| 27 | 16 | INHBA_Human | Inhibin beta A chain | 0.010 | 0.280 | 28.0 |
| 7 | 3 | HSPB1_Human | Heat shock protein beta-1 | 0.000 | 0.012 | 28.0 |
| 26 | 13 | LDHA_Human | L-lactate dehydrogenase A chain | 0.026 | 0.713 | 27.9 |
| 2 | 2 | ARC1B_Human | Actin-related protein 2/3 complex subunit 1B | 0.000 | 0.006 | 27.8 |

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| 12 | 7 | AMD_Human | Peptidyl-glycine alpha-amidating monooxygenase | 0.001 | 0.030 | 27.7 |
| 1 | 1 | ROAA_Human | Heterogeneous nuclear ribonucleoprotein A/B | 0.000 | 0.005 | 27.6 |
| 8 | 5 | DPYL2_Human | Dihydropyrimidinase-related protein 2 | 0.001 | 0.016 | 27.5 |
| 3 | 2 | AN32B_Human | Acidic leucine-rich nuclear phosphoprotein 32 family member B | 0.000 | 0.005 | 27.4 |
| 16 | 12 | AMPB_Human | Aminopeptidase B | 0.002 | 0.065 | 27.4 |
| 6 | 5 | PSB2_Human | Proteasome subunit beta type-2 | 0.001 | 0.039 | 27.3 |
| 13 | 11 | CLC11_Human | C-type lectin domain family 11 member A | 0.003 | 0.081 | 27.2 |
| 16 | 12 | PCD10_Human | Protocadherin-10 | 0.002 | 0.054 | 27.2 |
| 5 | 2 | ECI1_Human | Enoyl-CoA delta isomerase 1, mitochondrial | 0.000 | 0.008 | 27.1 |
| 16 | 13 | MDHC_Human | Malate dehydrogenase, cytoplasmic | 0.007 | 0.189 | 27.0 |
| 9 | 6 | IBP5_Human | Insulin-like growth factor-binding protein 5 | 0.001 | 0.029 | 26.9 |
| 13 | 11 | TAGL2_Human | Transgelin-2 | 0.003 | 0.091 | 26.9 |
| 9 | 4 | ARP3_Human | Actin-related protein 3 | 0.001 | 0.015 | 26.8 |
| 10 | 9 | AK1A1_Human | Alcohol dehydrogenase [NADP(+)] | 0.001 | 0.033 | 26.7 |
| 10 | 6 | FSTL1_Human | Follistatin-related protein 1 | 0.001 | 0.025 | 26.6 |
| 1 | 1 | BAF_Human | Barrier-to-autointegration factor | 0.000 | 0.001 | 26.5 |
| 7 | 3 | UGPA_Human | UTP--glucose-1-phosphate uridylyltransferase | 0.000 | 0.007 | 26.4 |
| 1 | 1 | KAT3_Human | Kynurenine--oxoglutarate transaminase 3 | 0.000 | 0.002 | 26.2 |
| 15 | 11 | PRDX6_Human | Peroxiredoxin-6 | 0.003 | 0.090 | 26.2 |

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|---|---|---|---|---|---|---|
| 1 | 2 | GLO2_Human | Hydroxyacylglutathione hydrolase, mitochondrial {ECO:0000303\|PubMed:15117945} | 0.000 | 0.003 | 26.2 |
| 16 | 11 | DPP3_Human | Dipeptidyl peptidase 3 | 0.002 | 0.043 | 26.1 |
| 5 | 1 | ARF1_Human | ADP-ribosylation factor 1 | 0.000 | 0.002 | 26.0 |
| 21 | 6 | XYLT1_Human | Xylosyltransferase 1 | 0.001 | 0.028 | 25.9 |
| 2 | 1 | MA1B1_Human | Endoplasmic reticulum mannosyl-oligosaccharide 1,2-alpha-mannosidase | 0.000 | 0.002 | 25.9 |
| 1 | 1 | S10AG_Human | Protein S100-A16 | 0.000 | 0.001 | 25.8 |
| 4 | 2 | GALT6_Human | Polypeptide N-acetylgalactosaminyltransferase 6 | 0.000 | 0.005 | 25.8 |
| 10 | 7 | CATD_Human | Cathepsin D | 0.002 | 0.039 | 25.7 |
| 49 | 15 | VIME_Human | Vimentin | 0.029 | 0.750 | 25.6 |
| 7 | 6 | PDLI5_Human | PDZ and LIM domain protein 5 | 0.001 | 0.019 | 25.5 |
| 14 | 9 | HS71B_Human | Heat shock 70 kDa protein 1B {ECO:0000312\|HGNC:HGNC:5233} | 0.003 | 0.064 | 25.4 |
| 7 | 5 | ERAP1_Human | Endoplasmic reticulum aminopeptidase 1 | 0.000 | 0.011 | 25.4 |
| 5 | 4 | TXD17_Human | Thioredoxin domain-containing protein 17 | 0.001 | 0.033 | 25.4 |
| 2 | 1 | STC1_Human | Stanniocalcin-1 | 0.000 | 0.002 | 25.2 |
| 1 | 1 | CUTA_Human | Protein CutA | 0.000 | 0.004 | 25.2 |
| 15 | 14 | G3P_Human | Glyceraldehyde-3-phosphate dehydrogenase | 0.011 | 0.285 | 25.2 |
| 3 | 2 | SEPR_Human | Prolyl endopeptidase FAP {ECO:0000305} | 0.000 | 0.005 | 25.1 |
| 18 | 14 | TPIS_Human | Triosephosphate isomerase | 0.021 | 0.521 | 25.1 |
| 18 | 10 | TRXR1_Human | Thioredoxin reductase 1, cytoplasmic | 0.002 | 0.057 | 24.9 |
| 7 | 3 | ATL1_Human | ADAMTS-like protein 1 | 0.001 | 0.017 | 24.8 |

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| 4 | 2 | VP26A_Human | Vacuolar protein sorting-associated protein 26A | 0.000 | 0.007 | 24.7 |
| 1 | 1 | STRP1_Human | Striatin-interacting protein 1 | 0.000 | 0.001 | 24.6 |
| 1 | 1 | ERP44_Human | Endoplasmic reticulum resident protein 44 | 0.000 | 0.002 | 24.6 |
| 5 | 1 | PURA2_Human | Adenylosuccinate synthetase isozyme 2 {ECO:0000255\|HAMAP-Rule:MF_03127} | 0.000 | 0.002 | 24.6 |
| 13 | 6 | CBR1_Human | Carbonyl reductase [NADPH] 1 | 0.002 | 0.038 | 24.5 |
| 8 | 5 | GLU2B_Human | Glucosidase 2 subunit beta | 0.001 | 0.022 | 24.5 |
| 4 | 4 | PROF2_Human | Profilin-2 | 0.000 | 0.011 | 24.4 |
| 3 | 2 | TPM3_Human | Tropomyosin alpha-3 chain | 0.000 | 0.004 | 24.2 |
| 17 | 11 | SODM_Human | Superoxide dismutase [Mn], mitochondrial | 0.003 | 0.074 | 24.2 |
| 14 | 9 | PRDX1_Human | Peroxiredoxin-1 | 0.007 | 0.161 | 24.0 |
| 22 | 13 | WDR1_Human | WD repeat-containing protein 1 | 0.005 | 0.126 | 23.9 |
| 33 | 14 | EF2_Human | Elongation factor 2 | 0.006 | 0.145 | 23.9 |
| 9 | 6 | B4GA1_Human | Beta-1,4-glucuronyltransferase 1 {ECO:0000303\|PubMed:25279697, ECO:0000303\|PubMed:25279699, ECO:0000312\|HGNC:HGNC:15685} | 0.002 | 0.038 | 23.8 |
| 3 | 1 | U2AF2_Human | Splicing factor U2AF 65 kDa subunit | 0.000 | 0.004 | 23.8 |
| 9 | 3 | ARPC2_Human | Actin-related protein 2/3 complex subunit 2 | 0.001 | 0.018 | 23.6 |
| 11 | 8 | QPCT_Human | Glutaminyl-peptide cyclotransferase | 0.002 | 0.045 | 23.6 |
| 18 | 10 | ROA2_Human | Heterogeneous nuclear | 0.003 | 0.077 | 23.6 |

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| | | | ribonucleoproteins A2/B1 | | | |
| 1 | 1 | SEPT9_Human | Septin-9 | 0.000 | 0.002 | 23.6 |
| 6 | 4 | 6PGD_Human | 6-phosphogluconate dehydrogenase, decarboxylating | 0.001 | 0.014 | 23.5 |
| 13 | 9 | TALDO_Human | Transaldolase | 0.002 | 0.057 | 23.4 |
| 4 | 1 | CALU_Human | Calumenin | 0.000 | 0.009 | 23.3 |
| 8 | 4 | CNDP2_Human | Cytosolic non-specific dipeptidase | 0.001 | 0.017 | 23.1 |
| 1 | 1 | TFPI1_Human | Tissue factor pathway inhibitor | 0.000 | 0.002 | 23.1 |
| 12 | 11 | CYTC_Human | Cystatin-C | 0.015 | 0.341 | 23.0 |
| 2 | 2 | LXN_Human | Latexin | 0.000 | 0.007 | 22.7 |
| 6 | 4 | SRPX2_Human | Sushi repeat-containing protein SRPX2 | 0.001 | 0.021 | 22.6 |
| 23 | 12 | LRP1_Human | Prolow-density lipoprotein receptor-related protein 1 | 0.002 | 0.037 | 22.6 |
| 9 | 7 | PSA1_Human | Proteasome subunit alpha type-1 | 0.002 | 0.051 | 22.5 |
| 1 | 1 | HIBCH_Human | 3-hydroxyisobutyryl-CoA hydrolase, mitochondrial | 0.000 | 0.002 | 22.5 |
| 6 | 4 | PRDX3_Human | Thioredoxin-dependent peroxide reductase, mitochondrial | 0.001 | 0.017 | 22.4 |
| 5 | 3 | PVRL2_Human | Nectin-2 | 0.000 | 0.009 | 22.4 |
| 2 | 2 | CYTB_Human | Cystatin-B | 0.000 | 0.010 | 22.4 |
| 16 | 12 | PPIA_Human | Peptidyl-prolyl cis-trans isomerase A | 0.010 | 0.226 | 22.4 |
| 2 | 2 | 1A66_Human | HLA class I histocompatibility antigen, A-66 alpha chain | 0.000 | 0.004 | 22.4 |
| 11 | 7 | IL6_Human | Interleukin-6 | 0.006 | 0.140 | 22.4 |
| 3 | 2 | KAD1_Human | Adenylate kinase isoenzyme 1 {ECO:0000255\|HAMAP-Rule:MF_03171} | 0.000 | 0.006 | 22.4 |
| 42 | 10 | PLEC_Human | Plectin | 0.002 | 0.043 | 22.3 |
| 10 | 10 | CH60_Human | 60 kDa heat shock protein, mitochondrial | 0.001 | 0.032 | 22.3 |

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|---|---|---|---|---|---|---|
| 3 | 3 | SYK_Human | Lysine--tRNA ligase | 0.000 | 0.007 | 22.3 |
| 3 | 1 | LDLR_Human | Low-density lipoprotein receptor | 0.000 | 0.003 | 22.2 |
| 6 | 3 | CAN2_Human | Calpain-2 catalytic subunit | 0.000 | 0.007 | 22.1 |
| 3 | 2 | TPP1_Human | Tripeptidyl-peptidase 1 | 0.000 | 0.002 | 22.1 |
| 10 | 8 | CLIC1_Human | Chloride intracellular channel protein 1 | 0.003 | 0.076 | 22.1 |
| 2 | 1 | BPNT1_Human | 3'(2'),5'-bisphosphate nucleotidase 1 | 0.000 | 0.003 | 22.0 |
| 2 | 2 | ITB1_Human | Integrin beta-1 | 0.000 | 0.004 | 22.0 |
| 17 | 11 | EXT1_Human | Exostosin-1 | 0.003 | 0.068 | 21.9 |
| 29 | 12 | TLN1_Human | Talin-1 | 0.002 | 0.046 | 21.9 |
| 12 | 10 | CLUS_Human | Clusterin | 0.004 | 0.088 | 21.8 |
| 6 | 5 | PSB3_Human | Proteasome subunit beta type-3 | 0.001 | 0.027 | 21.7 |
| 7 | 7 | FUMH_Human | Fumarate hydratase, mitochondrial | 0.001 | 0.024 | 21.6 |
| 3 | 2 | CAPR1_Human | Caprin-1 | 0.000 | 0.005 | 21.6 |
| 13 | 11 | ALDR_Human | Aldose reductase | 0.005 | 0.101 | 21.5 |
| 6 | 4 | SDCB1_Human | Syntenin-1 | 0.001 | 0.012 | 21.5 |
| 2 | 2 | ILF3_Human | Interleukin enhancer-binding factor 3 | 0.000 | 0.008 | 21.4 |
| 2 | 2 | TPM1_Human | Tropomyosin alpha-1 chain | 0.000 | 0.004 | 21.4 |
| 7 | 3 | TPM4_Human | Tropomyosin alpha-4 chain | 0.000 | 0.010 | 21.3 |
| 24 | 10 | ACTG_Human | Actin, cytoplasmic 2 | 0.011 | 0.235 | 21.2 |
| 14 | 12 | MYH9_Human | Myosin-9 | 0.003 | 0.055 | 21.2 |
| 97 | 14 | PGBM_Human | Basement membrane-specific heparan sulfate proteoglycan core protein | 0.013 | 0.267 | 21.1 |
| 7 | 6 | IF6_Human | Eukaryotic translation initiation factor 6 {ECO:0000255\|HAMAP-Rule:MF_03132} | 0.001 | 0.018 | 21.1 |
| 9 | 8 | IPO5_Human | Importin-5 | 0.001 | 0.023 | 21.1 |

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|---|---|---|---|---|---|---|
| 38 | 13 | CSPG4_Human | Chondroitin sulfate proteoglycan 4 | 0.004 | 0.084 | 21.1 |
| 9 | 7 | MXRA8_Human | Matrix-remodeling-associated protein 8 | 0.002 | 0.049 | 21.0 |
| 19 | 13 | CADH2_Human | Cadherin-2 | 0.005 | 0.103 | 21.0 |
| 16 | 9 | CAP1_Human | Adenylyl cyclase-associated protein 1 | 0.004 | 0.092 | 21.0 |
| 4 | 2 | TFPI2_Human | Tissue factor pathway inhibitor 2 | 0.000 | 0.008 | 21.0 |
| 4 | 1 | 1A69_Human | HLA class I histocompatibility antigen, A-69 alpha chain | 0.000 | 0.001 | 20.9 |
| 10 | 4 | SPB7_Human | Serpin B7 | 0.001 | 0.019 | 20.8 |
| 5 | 3 | ERP29_Human | Endoplasmic reticulum resident protein 29 | 0.000 | 0.009 | 20.8 |
| 15 | 11 | CLIC4_Human | Chloride intracellular channel protein 4 | 0.003 | 0.064 | 20.7 |
| 4 | 3 | T132A_Human | Transmembrane protein 132A | 0.000 | 0.006 | 20.7 |
| 4 | 1 | COPG1_Human | Coatomer subunit gamma-1 | 0.000 | 0.005 | 20.7 |
| 3 | 1 | BCAT1_Human | Branched-chain-amino-acid aminotransferase, cytosolic | 0.000 | 0.004 | 20.7 |
| 2 | 2 | AP2A1_Human | AP-2 complex subunit alpha-1 | 0.000 | 0.005 | 20.7 |
| 3 | 1 | GALNS_Human | N-acetylgalactosamine-6-sulfatase | 0.000 | 0.002 | 20.6 |
| 1 | 1 | PP2BA_Human | Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform | 0.000 | 0.002 | 20.6 |
| 16 | 13 | PPIB_Human | Peptidyl-prolyl cis-trans isomerase B | 0.024 | 0.495 | 20.4 |
| 2 | 2 | PDGFD_Human | Platelet-derived growth factor D | 0.000 | 0.004 | 20.4 |
| 9 | 8 | CATL1_Human | Cathepsin L1 | 0.002 | 0.038 | 20.4 |
| 7 | 3 | GRP75_Human | Stress-70 protein, mitochondrial | 0.000 | 0.010 | 20.3 |
| 1 | 1 | HEM2_Human | Delta-aminolevulinic acid dehydratase | 0.000 | 0.003 | 20.2 |

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|---|---|---|---|---|---|---|
| 5 | 2 | UB2V1_Human | Ubiquitin-conjugating enzyme E2 variant 1 | 0.001 | 0.011 | 20.1 |
| 7 | 2 | LAMA2_Human | Laminin subunit alpha-2 | 0.000 | 0.003 | 20.1 |
| 7 | 7 | CSRP1_Human | Cysteine and glycine-rich protein 1 | 0.002 | 0.040 | 20.1 |
| 6 | 3 | GLT10_Human | Polypeptide N-acetylgalactosaminyltransferase 10 | 0.001 | 0.013 | 20.0 |
| 4 | 4 | HNRPC_Human | Heterogeneous nuclear ribonucleoproteins C1/C2 | 0.002 | 0.030 | 19.9 |
| 9 | 6 | EZRI_Human | Ezrin | 0.001 | 0.021 | 19.8 |
| 14 | 10 | NEO1_Human | Neogenin | 0.002 | 0.037 | 19.8 |
| 15 | 14 | SERC_Human | Phosphoserine aminotransferase | 0.005 | 0.099 | 19.8 |
| 2 | 2 | MLP3B_Human | Microtubule-associated proteins 1A/1B light chain 3B | 0.001 | 0.013 | 19.8 |
| 8 | 6 | GLOD4_Human | Glyoxalase domain-containing protein 4 | 0.001 | 0.025 | 19.7 |
| 1 | 1 | PUF60_Human | Poly(U)-binding-splicing factor PUF60 | 0.000 | 0.001 | 19.7 |
| 11 | 8 | CAB45_Human | 45 kDa calcium-binding protein | 0.002 | 0.035 | 19.6 |
| 6 | 3 | THIM_Human | 3-ketoacyl-CoA thiolase, mitochondrial | 0.001 | 0.011 | 19.5 |
| 8 | 6 | PRDX2_Human | Peroxiredoxin-2 | 0.002 | 0.043 | 19.4 |
| 7 | 4 | ATPB_Human | ATP synthase subunit beta, mitochondrial | 0.001 | 0.014 | 19.4 |
| 8 | 5 | DEST_Human | Destrin | 0.003 | 0.063 | 19.4 |
| 5 | 2 | COR1C_Human | Coronin-1C | 0.001 | 0.010 | 19.4 |
| 3 | 1 | FA49B_Human | Protein FAM49B | 0.000 | 0.002 | 19.3 |
| 18 | 13 | TIMP2_Human | Metalloproteinase inhibitor 2 | 0.011 | 0.217 | 19.3 |
| 2 | 1 | RD23B_Human | UV excision repair protein RAD23 homolog B | 0.000 | 0.004 | 19.2 |

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|---|---|---|---|---|---|---|
| 3 | 3 | FKBP3_Human | Peptidyl-prolyl cis-trans isomerase FKBP3 | 0.000 | 0.008 | 19.2 |
| 6 | 6 | GDIR1_Human | Rho GDP-dissociation inhibitor 1 | 0.004 | 0.073 | 19.1 |
| 28 | 11 | FBN2_Human | Fibrillin-2 | 0.002 | 0.031 | 19.1 |
| 1 | 2 | ZYX_Human | Zyxin | 0.000 | 0.003 | 18.9 |
| 3 | 3 | SCRN1_Human | Secernin-1 | 0.000 | 0.007 | 18.9 |
| 6 | 5 | FLRT2_Human | Leucine-rich repeat transmembrane protein FLRT2 | 0.001 | 0.018 | 18.8 |
| 5 | 4 | GDF15_Human | Growth/differentiation factor 15 | 0.001 | 0.015 | 18.8 |
| 13 | 7 | LEG1_Human | Galectin-1 | 0.006 | 0.122 | 18.7 |
| 17 | 6 | PLOD1_Human | Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 | 0.001 | 0.022 | 18.7 |
| 5 | 2 | PABP1_Human | Polyadenylate-binding protein 1 | 0.000 | 0.006 | 18.6 |
| 2 | 1 | PA1B2_Human | Platelet-activating factor acetylhydrolase IB subunit beta | 0.000 | 0.003 | 18.6 |
| 14 | 9 | 1433Z_Human | 14-3-3 protein zeta/delta | 0.008 | 0.153 | 18.6 |
| 7 | 4 | PLIN3_Human | Perilipin-3 | 0.001 | 0.017 | 18.5 |
| 1 | 1 | APOA1_Human | Apolipoprotein A-I | 0.000 | 0.003 | 18.5 |
| 16 | 11 | NRP1_Human | Neuropilin-1 | 0.003 | 0.051 | 18.5 |
| 10 | 7 | 6PGL_Human | 6-phosphogluconolactonase | 0.003 | 0.054 | 18.5 |
| 3 | 2 | NEUS_Human | Neuroserpin | 0.000 | 0.004 | 18.4 |
| 3 | 1 | RS28_Human | 40S ribosomal protein S28 | 0.001 | 0.009 | 18.3 |
| 3 | 1 | SAHH_Human | Adenosylhomocysteinase | 0.000 | 0.002 | 18.3 |
| 12 | 7 | GSTO1_Human | Glutathione S-transferase omega-1 | 0.006 | 0.111 | 18.3 |
| 5 | 2 | IMPA1_Human | Inositol monophosphatase 1 | 0.000 | 0.005 | 18.3 |
| 2 | 1 | RCN1_Human | Reticulocalbin-1 | 0.000 | 0.002 | 18.2 |
| 6 | 5 | UBE2N_Human | Ubiquitin-conjugating enzyme E2 N | 0.002 | 0.034 | 18.2 |

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|---|---|---|---|---|---|---|
| 6 | 3 | ILF2_Human | Interleukin enhancer-binding factor 2 | 0.001 | 0.016 | 18.1 |
| 3 | 2 | METK2_Human | S-adenosylmethionine synthase isoform type-2 | 0.000 | 0.006 | 18.0 |
| 8 | 6 | IF5A1_Human | Eukaryotic translation initiation factor 5A-1 | 0.001 | 0.024 | 18.0 |
| 25 | 11 | FLNC_Human | Filamin-C | 0.002 | 0.038 | 18.0 |
| 6 | 5 | CYC_Human | Cytochrome c | 0.004 | 0.064 | 17.9 |
| 11 | 8 | PSB5_Human | Proteasome subunit beta type-5 | 0.002 | 0.036 | 17.8 |
| 5 | 5 | 1433B_Human | 14-3-3 protein beta/alpha | 0.003 | 0.049 | 17.8 |
| 3 | 1 | SYG_Human | Glycine--tRNA ligase | 0.000 | 0.001 | 17.8 |
| 3 | 3 | LAMB2_Human | Laminin subunit beta-2 | 0.000 | 0.006 | 17.8 |
| 2 | 1 | IFT25_Human | Intraflagellar transport protein 25 homolog | 0.000 | 0.003 | 17.6 |
| 30 | 14 | LMNA_Human | Prelamin-A/C | 0.007 | 0.126 | 17.6 |
| 11 | 8 | CTGF_Human | Connective tissue growth factor | 0.003 | 0.049 | 17.6 |
| 4 | 3 | ANAG_Human | Alpha-N-acetylglucosaminidase | 0.000 | 0.006 | 17.5 |
| 17 | 11 | PSA_Human | Puromycin-sensitive aminopeptidase | 0.002 | 0.041 | 17.5 |
| 5 | 4 | FKBP4_Human | Peptidyl-prolyl cis-trans isomerase FKBP4 | 0.000 | 0.008 | 17.5 |
| 13 | 11 | COTL1_Human | Coactosin-like protein | 0.016 | 0.276 | 17.5 |
| 2 | 2 | RO60_Human | 60 kDa SS-A/Ro ribonucleoprotein | 0.000 | 0.005 | 17.4 |
| 4 | 1 | NTF2_Human | Nuclear transport factor 2 | 0.000 | 0.004 | 17.4 |
| 11 | 8 | PSA4_Human | Proteasome subunit alpha type-4 | 0.002 | 0.043 | 17.3 |
| 3 | 3 | RINI_Human | Ribonuclease inhibitor | 0.000 | 0.007 | 17.3 |
| 11 | 7 | PARK7_Human | Protein deglycase DJ-1 {ECO:0000303\|PubMed:25416785} | 0.004 | 0.077 | 17.3 |

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|---|---|---|---|---|---|---|
| 12 | 11 | AATC_Human | Aspartate aminotransferase, cytoplasmic | 0.003 | 0.058 | 17.3 |
| 6 | 5 | 1433T_Human | 14-3-3 protein theta | 0.001 | 0.021 | 17.1 |
| 5 | 3 | MARE1_Human | Microtubule-associated protein RP/EB family member 1 | 0.000 | 0.007 | 17.0 |
| 2 | 1 | MMP14_Human | Matrix metalloproteinase-14 | 0.000 | 0.001 | 17.0 |
| 3 | 1 | BLVRB_Human | Flavin reductase (NADPH) | 0.000 | 0.001 | 17.0 |
| 2 | 2 | MT1X_Human | Metallothionein-1X | 0.000 | 0.003 | 17.0 |
| 2 | 1 | CAPG_Human | Macrophage-capping protein | 0.000 | 0.002 | 16.9 |
| 6 | 5 | FAAA_Human | Fumarylacetoacetase | 0.001 | 0.021 | 16.9 |
| 3 | 2 | PCNA_Human | Proliferating cell nuclear antigen | 0.000 | 0.004 | 16.9 |
| 3 | 1 | NNRE_Human | NAD(P)H-hydrate epimerase {ECO:0000255\|HAMAP-Rule:MF_03159} | 0.000 | 0.003 | 16.9 |
| 5 | 2 | COEA1_Human | Collagen alpha-1(XIV) chain | 0.001 | 0.009 | 16.9 |
| 7 | 5 | RL40_Human | Ubiquitin-60S ribosomal protein L40 | 0.014 | 0.242 | 16.7 |
| 3 | 2 | EF1D_Human | Elongation factor 1-delta | 0.001 | 0.012 | 16.6 |
| 5 | 1 | PDLI7_Human | PDZ and LIM domain protein 7 | 0.001 | 0.010 | 16.5 |
| 1 | 1 | ROA3_Human | Heterogeneous nuclear ribonucleoprotein A3 | 0.000 | 0.003 | 16.4 |
| 5 | 4 | DKK1_Human | Dickkopf-related protein 1 | 0.001 | 0.018 | 16.4 |
| 10 | 9 | MATN2_Human | Matrilin-2 | 0.002 | 0.030 | 16.4 |
| 1 | 1 | ITA2_Human | Integrin alpha-2 | 0.000 | 0.001 | 16.3 |
| 9 | 7 | PSA2_Human | Proteasome subunit alpha type-2 | 0.003 | 0.041 | 16.2 |
| 16 | 10 | LAMC2_Human | Laminin subunit gamma-2 | 0.002 | 0.034 | 16.2 |
| 3 | 3 | CBPQ_Human | Carboxypeptidase Q | 0.000 | 0.007 | 16.1 |
| 7 | 4 | TSN_Human | Translin | 0.001 | 0.009 | 16.1 |

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|---|---|---|---|---|---|---|
| 4 | 2 | MAP1B_Human | Microtubule-associated protein 1B | 0.000 | 0.005 | 16.1 |
| 13 | 6 | CO7A1_Human | Collagen alpha-1(VII) chain | 0.001 | 0.015 | 16.1 |
| 19 | 15 | AATM_Human | Aspartate aminotransferase, mitochondrial | 0.011 | 0.172 | 16.0 |
| 1 | 1 | N2DL2_Human | NKG2D ligand 2 | 0.000 | 0.003 | 15.9 |
| 10 | 7 | CCD80_Human | Coiled-coil domain-containing protein 80 | 0.002 | 0.031 | 15.9 |
| 5 | 3 | ILEU_Human | Leukocyte elastase inhibitor | 0.000 | 0.005 | 15.8 |
| 2 | 2 | MAT2B_Human | Methionine adenosyltransferase 2 subunit beta | 0.000 | 0.003 | 15.8 |
| 1 | 1 | SMD1_Human | Small nuclear ribonucleoprotein Sm D1 | 0.000 | 0.003 | 15.8 |
| 3 | 3 | YBOX1_Human | Nuclease-sensitive element-binding protein 1 | 0.001 | 0.009 | 15.8 |
| 4 | 2 | FAM3C_Human | Protein FAM3C | 0.000 | 0.004 | 15.8 |
| 3 | 2 | CSTN3_Human | Calsyntenin-3 | 0.000 | 0.004 | 15.7 |
| 4 | 3 | SH3L3_Human | SH3 domain-binding glutamic acid-rich-like protein 3 | 0.003 | 0.042 | 15.6 |
| 3 | 3 | HS90A_Human | Heat shock protein HSP 90-alpha | 0.001 | 0.009 | 15.6 |
| 6 | 5 | CXCL6_Human | C-X-C motif chemokine 6 | 0.006 | 0.096 | 15.6 |
| 10 | 5 | HNRPQ_Human | Heterogeneous nuclear ribonucleoprotein Q | 0.001 | 0.016 | 15.6 |
| 9 | 3 | RENR_Human | Renin receptor | 0.000 | 0.006 | 15.6 |
| 2 | 1 | IF2A_Human | Eukaryotic translation initiation factor 2 subunit 1 | 0.000 | 0.003 | 15.5 |
| 6 | 5 | APEX1_Human | DNA-(apurinic or apyrimidinic site) lyase | 0.001 | 0.011 | 15.4 |
| 1 | 1 | MFAP4_Human | Microfibril-associated glycoprotein 4 | 0.000 | 0.002 | 15.4 |

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|---|---|---|---|---|---|---|
| 6 | 4 | COPE_Human | Coatomer subunit epsilon | 0.001 | 0.009 | 15.4 |
| 6 | 2 | MANF_Human | Mesencephalic astrocyte-derived neurotrophic factor | 0.000 | 0.004 | 15.2 |
| 7 | 3 | CPNS1_Human | Calpain small subunit 1 | 0.001 | 0.015 | 15.1 |
| 1 | 1 | SPEE_Human | Spermidine synthase | 0.000 | 0.003 | 15.1 |
| 3 | 2 | RBM8A_Human | RNA-binding protein 8A | 0.000 | 0.005 | 15.1 |
| 9 | 7 | CANT1_Human | Soluble calcium-activated nucleotidase 1 | 0.001 | 0.021 | 15.1 |
| 7 | 4 | ARPC4_Human | Actin-related protein 2/3 complex subunit 4 | 0.002 | 0.030 | 15.0 |
| 4 | 4 | RL12_Human | 60S ribosomal protein L12 | 0.001 | 0.022 | 15.0 |
| 1 | 1 | TBL1R_Human | F-box-like/WD repeat-containing protein TBL1XR1 | 0.000 | 0.001 | 15.0 |
| 8 | 5 | TGFB1_Human | Transforming growth factor beta-1 | 0.001 | 0.020 | 14.9 |
| 1 | 1 | SEP11_Human | Septin-11 | 0.000 | 0.002 | 14.9 |
| 12 | 4 | NDKB_Human | Nucleoside diphosphate kinase B | 0.003 | 0.042 | 14.9 |
| 3 | 3 | CAN1_Human | Calpain-1 catalytic subunit | 0.001 | 0.008 | 14.8 |
| 2 | 1 | SPB9_Human | Serpin B9 | 0.000 | 0.002 | 14.7 |
| 2 | 1 | PPGB_Human | Lysosomal protective protein | 0.000 | 0.001 | 14.7 |
| 1 | 1 | NUDT5_Human | ADP-sugar pyrophosphatase | 0.000 | 0.003 | 14.7 |
| 2 | 1 | IL11_Human | Interleukin-11 | 0.000 | 0.002 | 14.7 |
| 1 | 1 | EFHD2_Human | EF-hand domain-containing protein D2 | 0.000 | 0.002 | 14.6 |
| 4 | 2 | TCPQ_Human | T-complex protein 1 subunit theta | 0.000 | 0.005 | 14.6 |
| 14 | 12 | SPB6_Human | Serpin B6 | 0.006 | 0.087 | 14.6 |
| 11 | 6 | ACPH_Human | Acylamino-acid-releasing enzyme | 0.001 | 0.020 | 14.5 |
| 11 | 9 | ANXA2_Human | Annexin A2 | 0.002 | 0.025 | 14.4 |
| 15 | 9 | PROF1_Human | Profilin-1 | 0.057 | 0.813 | 14.3 |
| 23 | 12 | GELS_Human | Gelsolin | 0.004 | 0.051 | 14.3 |

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|---|---|---|---|---|---|---|
| 11 | 8 | PSA6_Human | Proteasome subunit alpha type-6 | 0.003 | 0.039 | 14.3 |
| 6 | 2 | MPRI_Human | Cation-independent mannose-6-phosphate receptor | 0.000 | 0.005 | 14.2 |
| 5 | 4 | KCY_Human | UMP-CMP kinase {ECO:0000255\|HAMAP-Rule:MF_03172} | 0.001 | 0.011 | 14.2 |
| 11 | 10 | PSA7_Human | Proteasome subunit alpha type-7 | 0.005 | 0.076 | 14.2 |
| 10 | 5 | B2MG_Human | Beta-2-microglobulin | 0.017 | 0.236 | 14.2 |
| 1 | 1 | CHSTE_Human | Carbohydrate sulfotransferase 14 | 0.000 | 0.002 | 14.2 |
| 1 | 1 | MXRA5_Human | Matrix-remodeling-associated protein 5 | 0.000 | 0.001 | 14.2 |
| 6 | 2 | COF2_Human | Cofilin-2 | 0.001 | 0.010 | 14.1 |
| 2 | 1 | ITM2B_Human | Integral membrane protein 2B | 0.000 | 0.002 | 14.1 |
| 6 | 3 | PRDX4_Human | Peroxiredoxin-4 | 0.001 | 0.010 | 14.1 |
| 4 | 2 | UBP14_Human | Ubiquitin carboxyl-terminal hydrolase 14 | 0.000 | 0.005 | 14.0 |
| 5 | 1 | VEGFC_Human | Vascular endothelial growth factor C | 0.000 | 0.003 | 14.0 |
| 2 | 1 | ANXA6_Human | Annexin A6 | 0.000 | 0.001 | 13.9 |
| 2 | 1 | ADA12_Human | Disintegrin and metalloproteinase domain-containing protein 12 | 0.000 | 0.003 | 13.9 |
| 38 | 11 | TENA_Human | Tenascin | 0.004 | 0.058 | 13.9 |
| 4 | 3 | ARPC3_Human | Actin-related protein 2/3 complex subunit 3 | 0.001 | 0.011 | 13.9 |
| 4 | 4 | ACTS_Human | Actin, alpha skeletal muscle | 0.003 | 0.048 | 13.8 |
| 10 | 8 | PEBP1_Human | Phosphatidylethanolamine-binding protein 1 | 0.007 | 0.095 | 13.8 |
| 5 | 4 | RLA0_Human | 60S acidic ribosomal protein P0 | 0.001 | 0.017 | 13.7 |
| 5 | 5 | VAT1_Human | Synaptic vesicle membrane protein VAT-1 homolog | 0.001 | 0.014 | 13.7 |
| 2 | 2 | DAG1_Human | Dystroglycan | 0.000 | 0.005 | 13.6 |

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|---|---|---|---|---|---|---|
| 2 | 1 | CAZA1_Human | F-actin-capping protein subunit alpha-1 | 0.000 | 0.005 | 13.6 |
| 3 | 2 | PICAL_Human | Phosphatidylinositol-binding clathrin assembly protein | 0.000 | 0.003 | 13.6 |
| 2 | 1 | H2AJ_Human | Histone H2A.J | 0.000 | 0.002 | 13.5 |
| 4 | 2 | SC31A_Human | Protein transport protein Sec31A | 0.000 | 0.004 | 13.5 |
| 5 | 4 | NUCL_Human | Nucleolin | 0.001 | 0.015 | 13.5 |
| 1 | 1 | RHG01_Human | Rho GTPase-activating protein 1 | 0.000 | 0.001 | 13.4 |
| 2 | 1 | PCDGK_Human | Protocadherin gamma-C3 | 0.000 | 0.001 | 13.3 |
| 9 | 3 | HPRT_Human | Hypoxanthine-guanine phosphoribosyltransferase | 0.001 | 0.008 | 13.3 |
| 1 | 1 | EPN3_Human | Epsin-3 | 0.000 | 0.001 | 13.2 |
| 14 | 8 | COF1_Human | Cofilin-1 | 0.012 | 0.153 | 13.2 |
| 3 | 2 | CIRBP_Human | Cold-inducible RNA-binding protein | 0.000 | 0.005 | 13.2 |
| 2 | 2 | RADI_Human | Radixin | 0.000 | 0.004 | 13.2 |
| 2 | 2 | VAPA_Human | Vesicle-associated membrane protein-associated protein A | 0.000 | 0.005 | 13.2 |
| 6 | 3 | 1433F_Human | 14-3-3 protein eta | 0.001 | 0.010 | 13.2 |
| 13 | 10 | EF1G_Human | Elongation factor 1-gamma | 0.005 | 0.060 | 13.1 |
| 7 | 5 | CHID1_Human | Chitinase domain-containing protein 1 | 0.001 | 0.015 | 13.1 |
| 9 | 5 | 1433G_Human | 14-3-3 protein gamma | 0.004 | 0.048 | 13.1 |
| 10 | 8 | PSA5_Human | Proteasome subunit alpha type-5 | 0.003 | 0.039 | 13.1 |
| 9 | 7 | TWF2_Human | Twinfilin-2 | 0.001 | 0.019 | 13.1 |
| 9 | 8 | IBP4_Human | Insulin-like growth factor-binding protein 4 | 0.005 | 0.066 | 13.1 |
| 2 | 2 | MFGM_Human | Lactadherin | 0.000 | 0.005 | 13.0 |
| 4 | 1 | ORN_Human | Oligoribonuclease, mitochondrial | 0.000 | 0.006 | 13.0 |
| 2 | 2 | AMPL_Human | Cytosol aminopeptidase | 0.000 | 0.004 | 13.0 |
| 5 | 2 | NAGK_Human | N-acetyl-D-glucosamine kinase | 0.000 | 0.005 | 13.0 |
| 6 | 3 | LMNB2_Human | Lamin-B2 | 0.000 | 0.005 | 12.7 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 3 | 3 | SIL1_Human | Nucleotide exchange factor SIL1 | 0.001 | 0.007 | 12.7 |
| 15 | 8 | IQGA1_Human | Ras GTPase-activating-like protein IQGAP1 | 0.002 | 0.023 | 12.6 |
| 1 | 1 | SLIT2_Human | Slit homolog 2 protein | 0.000 | 0.001 | 12.6 |
| 4 | 2 | CNN3_Human | Calponin-3 | 0.000 | 0.003 | 12.6 |
| 2 | 1 | RL5_Human | 60S ribosomal protein L5 | 0.000 | 0.001 | 12.6 |
| 4 | 2 | HEBP2_Human | Heme-binding protein 2 | 0.000 | 0.005 | 12.5 |
| 4 | 1 | PTPRS_Human | Receptor-type tyrosine-protein phosphatase S | 0.000 | 0.002 | 12.4 |
| 15 | 12 | LAMA5_Human | Laminin subunit alpha-5 | 0.002 | 0.023 | 12.3 |
| 12 | 2 | HS90B_Human | Heat shock protein HSP 90-beta | 0.001 | 0.012 | 12.3 |
| 2 | 2 | PARVA_Human | Alpha-parvin | 0.000 | 0.003 | 12.3 |
| 1 | 1 | XYLT2_Human | Xylosyltransferase 2 | 0.000 | 0.002 | 12.3 |
| 2 | 1 | TMOD3_Human | Tropomodulin-3 | 0.000 | 0.001 | 12.3 |
| 10 | 2 | PYGB_Human | Glycogen phosphorylase, brain form | 0.000 | 0.003 | 12.0 |
| 1 | 2 | GRPE1_Human | GrpE protein homolog 1, mitochondrial | 0.000 | 0.003 | 12.0 |
| 3 | 2 | FKB1A_Human | Peptidyl-prolyl cis-trans isomerase FKBP1A | 0.001 | 0.009 | 11.9 |
| 5 | 2 | SH3L1_Human | SH3 domain-binding glutamic acid-rich-like protein | 0.001 | 0.009 | 11.8 |
| 6 | 4 | QORX_Human | Quinone oxidoreductase PIG3 | 0.002 | 0.022 | 11.8 |
| 4 | 2 | COIA1_Human | Collagen alpha-1(XVIII) chain | 0.001 | 0.009 | 11.8 |
| 1 | 1 | UFD1_Human | Ubiquitin fusion degradation protein 1 homolog | 0.000 | 0.003 | 11.7 |
| 1 | 1 | RL30_Human | 60S ribosomal protein L30 | 0.000 | 0.001 | 11.7 |

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|---|---|---|---|---|---|---|
| 17 | 14 | PGAM1_Human | Phosphoglycerate mutase 1 | 0.014 | 0.160 | 11.6 |
| 7 | 3 | QOR_Human | Quinone oxidoreductase | 0.001 | 0.010 | 11.6 |
| 4 | 2 | G6PD_Human | Glucose-6-phosphate 1-dehydrogenase | 0.000 | 0.005 | 11.6 |
| 1 | 1 | SND1_Human | Staphylococcal nuclease domain-containing protein 1 | 0.000 | 0.003 | 11.5 |
| 2 | 2 | CNN2_Human | Calponin-2 | 0.000 | 0.004 | 11.5 |
| 3 | 2 | VTNC_Human | Vitronectin | 0.002 | 0.020 | 11.5 |
| 3 | 2 | UBP5_Human | Ubiquitin carboxyl-terminal hydrolase 5 | 0.000 | 0.003 | 11.5 |
| 8 | 3 | PSB7_Human | Proteasome subunit beta type-7 | 0.002 | 0.020 | 11.4 |
| 6 | 3 | CH10_Human | 10 kDa heat shock protein, mitochondrial | 0.001 | 0.011 | 11.3 |
| 4 | 2 | GNPI1_Human | Glucosamine-6-phosphate isomerase 1 | 0.001 | 0.007 | 11.3 |
| 6 | 2 | PTPRF_Human | Receptor-type tyrosine-protein phosphatase F | 0.000 | 0.004 | 11.2 |
| 5 | 3 | PSB6_Human | Proteasome subunit beta type-6 | 0.002 | 0.020 | 11.2 |
| 3 | 1 | ARL3_Human | ADP-ribosylation factor-like protein 3 | 0.000 | 0.001 | 11.2 |
| 2 | 2 | CRIM1_Human | Cysteine-rich motor neuron 1 protein | 0.000 | 0.002 | 11.2 |
| 2 | 1 | PHS_Human | Pterin-4-alpha-carbinolamine dehydratase | 0.000 | 0.003 | 10.9 |
| 3 | 2 | SODC_Human | Superoxide dismutase [Cu-Zn] | 0.000 | 0.005 | 10.8 |
| 5 | 3 | VGFR1_Human | Vascular endothelial growth factor receptor 1 | 0.001 | 0.008 | 10.8 |
| 1 | 1 | C1QT1_Human | Complement C1q tumor necrosis factor-related protein 1 | 0.000 | 0.002 | 10.7 |
| 3 | 2 | LRC59_Human | Leucine-rich repeat-containing protein 59 | 0.000 | 0.003 | 10.7 |
| 6 | 3 | UBE2K_Human | Ubiquitin-conjugating enzyme E2 K | 0.001 | 0.009 | 10.7 |

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|---|---|---|---|---|---|---|
| 3 | 1 | GSHR_Human | Glutathione reductase, mitochondrial | 0.000 | 0.002 | 10.7 |
| 4 | 2 | FST_Human | Follistatin | 0.000 | 0.004 | 10.6 |
| 3 | 2 | ESYT1_Human | Extended synaptotagmin-1 | 0.000 | 0.003 | 10.5 |
| 7 | 2 | ML12A_Human | Myosin regulatory light chain 12A | 0.000 | 0.005 | 10.5 |
| 6 | 7 | DDB1_Human | DNA damage-binding protein 1 | 0.002 | 0.021 | 10.4 |
| 3 | 1 | UFC1_Human | Ubiquitin-fold modifier-conjugating enzyme 1 | 0.000 | 0.003 | 10.3 |
| 3 | 2 | PCBP1_Human | Poly(rC)-binding protein 1 | 0.001 | 0.006 | 10.3 |
| 1 | 1 | MATN3_Human | Matrilin-3 | 0.000 | 0.002 | 10.3 |
| 13 | 6 | COPD_Human | Coatomer subunit delta | 0.002 | 0.021 | 10.3 |
| 10 | 6 | PSB1_Human | Proteasome subunit beta type-1 | 0.003 | 0.034 | 10.3 |
| 2 | 1 | IBP6_Human | Insulin-like growth factor-binding protein 6 | 0.000 | 0.003 | 10.2 |
| 2 | 1 | MTAP_Human | S-methyl-5'-thioadenosine phosphorylase | 0.000 | 0.003 | 10.2 |
| 2 | 2 | SYQ_Human | Glutamine--tRNA ligase | 0.000 | 0.003 | 10.2 |
| 6 | 5 | TBB5_Human | Tubulin beta chain | 0.002 | 0.017 | 10.1 |
| 8 | 6 | PLTP_Human | Phospholipid transfer protein | 0.002 | 0.022 | 10.1 |
| 5 | 4 | CFAB_Human | Complement factor B | 0.002 | 0.018 | 10.0 |
| 3 | 3 | GNPTG_Human | N-acetylglucosamine-1-phosphotransferase subunit gamma | 0.001 | 0.008 | 10.0 |
| 1 | 1 | RS12_Human | 40S ribosomal protein S12 | 0.001 | 0.006 | 10.0 |
| 3 | 2 | NEUL_Human | Neurolysin, mitochondrial | 0.000 | 0.003 | 10.0 |
| 2 | 2 | PTGR1_Human | Prostaglandin reductase 1 | 0.000 | 0.004 | 9.9 |
| 2 | 1 | ANM1_Human | Protein arginine N-methyltransferase 1 | 0.000 | 0.002 | 9.7 |
| 3 | 1 | TFG_Human | Protein TFG | 0.000 | 0.004 | 9.7 |

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|---|---|---|---|---|---|---|
| 1 | 1 | TGM2_Human | Protein-glutamine gamma-glutamyltransferase 2 | 0.000 | 0.002 | 9.7 |
| 6 | 5 | MYL6_Human | Myosin light polypeptide 6 | 0.001 | 0.012 | 9.6 |
| 4 | 2 | RET1_Human | Retinol-binding protein 1 | 0.000 | 0.003 | 9.4 |
| 6 | 6 | K2C8_Human | Keratin, type II cytoskeletal 8 | 0.002 | 0.019 | 9.3 |
| 7 | 6 | PGM2_Human | Phosphoglucomutase-2 | 0.002 | 0.020 | 9.3 |
| 7 | 5 | K1C18_Human | Keratin, type I cytoskeletal 18 | 0.002 | 0.017 | 9.3 |
| 5 | 3 | ANXA1_Human | Annexin A1 | 0.001 | 0.005 | 9.2 |
| 3 | 1 | PAMR1_Human | Inactive serine protease PAMR1 | 0.000 | 0.001 | 9.2 |
| 3 | 3 | SYTC_Human | Threonine--tRNA ligase, cytoplasmic | 0.001 | 0.007 | 9.1 |
| 4 | 4 | P3H1_Human | Prolyl 3-hydroxylase 1 {ECO:0000312\|HGNC:HGNC:19316} | 0.001 | 0.012 | 9.1 |
| 2 | 1 | DTD1_Human | D-tyrosyl-tRNA(Tyr) deacylase 1 | 0.000 | 0.003 | 9.0 |
| 2 | 1 | S10AD_Human | Protein S100-A13 | 0.000 | 0.001 | 8.9 |
| 2 | 2 | LA_Human | Lupus La protein | 0.000 | 0.003 | 8.9 |
| 3 | 1 | DDAH2_Human | N(G),N(G)-dimethylarginine dimethylaminohydrolase 2 | 0.000 | 0.003 | 8.9 |
| 5 | 4 | S10AB_Human | Protein S100-A11 | 0.002 | 0.017 | 8.8 |
| 1 | 1 | SH24A_Human | SH2 domain-containing protein 4A | 0.001 | 0.005 | 8.8 |
| 2 | 2 | LEG3_Human | Galectin-3 | 0.000 | 0.004 | 8.8 |
| 1 | 1 | MMP3_Human | Stromelysin-1 | 0.000 | 0.003 | 8.8 |
| 2 | 1 | ENOG_Human | Gamma-enolase | 0.000 | 0.002 | 8.7 |
| 2 | 1 | LMNB1_Human | Lamin-B1 | 0.000 | 0.001 | 8.7 |
| 1 | 1 | PSB8_Human | Proteasome subunit beta type-8 | 0.000 | 0.003 | 8.6 |
| 2 | 2 | MYL9_Human | Myosin regulatory light polypeptide 9 | 0.000 | 0.003 | 8.5 |
| 8 | 6 | DSC3_Human | Desmocollin-3 | 0.006 | 0.054 | 8.5 |

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|---|---|---|---|---|---|---|
| 5 | 3 | FAHD1_Human | Acylpyruvase FAHD1, mitochondrial | 0.001 | 0.007 | 8.5 |
| 3 | 1 | ARSA_Human | Arylsulfatase A | 0.000 | 0.001 | 8.5 |
| 3 | 1 | COPB2_Human | Coatomer subunit beta' | 0.000 | 0.002 | 8.4 |
| 6 | 5 | RB11B_Human | Ras-related protein Rab-11B | 0.003 | 0.021 | 8.4 |
| 3 | 3 | IDI1_Human | Isopentenyl-diphosphate Delta-isomerase 1 | 0.000 | 0.004 | 8.3 |
| 4 | 3 | TSG6_Human | Tumor necrosis factor-inducible gene 6 protein | 0.002 | 0.017 | 8.2 |
| 5 | 2 | THIO_Human | Thioredoxin | 0.001 | 0.012 | 8.2 |
| 5 | 1 | MGT5A_Human | Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase A | 0.000 | 0.002 | 8.1 |
| 1 | 1 | SMD3_Human | Small nuclear ribonucleoprotein Sm D3 | 0.001 | 0.004 | 8.1 |
| 3 | 2 | SAP3_Human | Ganglioside GM2 activator | 0.001 | 0.005 | 8.1 |
| 1 | 1 | FIS1_Human | Mitochondrial fission 1 protein | 0.000 | 0.000 | 8.0 |
| 2 | 2 | FHL1_Human | Four and a half LIM domains protein 1 | 0.001 | 0.006 | 8.0 |
| 1 | 1 | TARA_Human | TRIO and F-actin-binding protein | 0.000 | 0.001 | 8.0 |
| 2 | 1 | HBD_Human | Hemoglobin subunit delta | 0.004 | 0.029 | 8.0 |
| 1 | 1 | ARF4_Human | ADP-ribosylation factor 4 | 0.000 | 0.001 | 7.9 |
| 2 | 2 | GNPI2_Human | Glucosamine-6-phosphate isomerase 2 | 0.000 | 0.002 | 7.8 |
| 3 | 3 | EF1B_Human | Elongation factor 1-beta | 0.001 | 0.008 | 7.8 |
| 2 | 2 | MTND_Human | 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase {ECO:0000255\|HAMAP-Rule:MF_03154} | 0.000 | 0.004 | 7.7 |
| 1 | 1 | ISLR_Human | Immunoglobulin superfamily | 0.000 | 0.001 | 7.7 |

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|---|---|---|---|---|---|---|
| | | | containing leucine-rich repeat protein | | | |
| 18 | 15 | CAD11_Human | Cadherin-11 | 0.021 | 0.162 | 7.7 |
| 2 | 2 | NIBL1_Human | Niban-like protein 1 | 0.001 | 0.004 | 7.7 |
| 3 | 1 | DCTN3_Human | Dynactin subunit 3 | 0.000 | 0.001 | 7.6 |
| 6 | 5 | FSTL5_Human | Follistatin-related protein 5 | 0.001 | 0.011 | 7.5 |
| 7 | 4 | GROA_Human | Growth-regulated alpha protein | 0.005 | 0.035 | 7.5 |
| 5 | 2 | ACOC_Human | Cytoplasmic aconitate hydratase | 0.001 | 0.004 | 7.4 |
| 4 | 1 | NPS3A_Human | Protein NipSnap homolog 3A | 0.000 | 0.002 | 7.3 |
| 1 | 1 | CDC42_Human | Cell division control protein 42 homolog | 0.000 | 0.001 | 7.2 |
| 4 | 3 | ITIH2_Human | Inter-alpha-trypsin inhibitor heavy chain H2 | 0.002 | 0.012 | 7.1 |
| 3 | 2 | LASP1_Human | LIM and SH3 domain protein 1 | 0.001 | 0.004 | 7.1 |
| 2 | 2 | HNRPR_Human | Heterogeneous nuclear ribonucleoprotein R | 0.000 | 0.003 | 7.1 |
| 13 | 5 | COBA1_Human | Collagen alpha-1(XI) chain | 0.002 | 0.013 | 7.0 |
| 4 | 4 | MGN2_Human | Protein mago nashi homolog 2 | 0.001 | 0.006 | 6.8 |
| 8 | 5 | AMPN_Human | Aminopeptidase N | 0.002 | 0.013 | 6.7 |
| 7 | 2 | HYI_Human | Putative hydroxypyruvate isomerase | 0.001 | 0.003 | 6.6 |
| 1 | 1 | UFM1_Human | Ubiquitin-fold modifier 1 | 0.000 | 0.003 | 6.6 |
| 3 | 2 | CNN1_Human | Calponin-1 | 0.000 | 0.003 | 6.4 |
| 2 | 1 | CDC37_Human | Hsp90 co-chaperone Cdc37 | 0.000 | 0.002 | 6.4 |
| 2 | 2 | MTPN_Human | Myotrophin | 0.000 | 0.002 | 6.4 |
| 1 | 1 | FAS_Human | Fatty acid synthase | 0.000 | 0.001 | 6.4 |
| 2 | 2 | THOP1_Human | Thimet oligopeptidase | 0.001 | 0.004 | 6.3 |
| 10 | 5 | A4_Human | Amyloid beta A4 protein | 0.003 | 0.017 | 6.3 |
| 1 | 1 | NQO2_Human | Ribosyldihydronicotinamide dehydrogenase [quinone] | 0.000 | 0.001 | 6.3 |
| 11 | 7 | TBA1B_Human | Tubulin alpha-1B chain | 0.004 | 0.025 | 6.2 |

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|---|---|---|---|---|---|---|
| 2 | 1 | 2AAA_Human | Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform | 0.000 | 0.001 | 6.1 |
| 13 | 6 | SPTN1_Human | Spectrin alpha chain, non-erythrocytic 1 | 0.001 | 0.006 | 6.0 |
| 2 | 1 | CPSF5_Human | Cleavage and polyadenylation specificity factor subunit 5 | 0.000 | 0.001 | 5.6 |
| 2 | 1 | PHP14_Human | 14 kDa phosphohistidine phosphatase | 0.000 | 0.001 | 5.6 |
| 1 | 1 | INPP_Human | Inositol polyphosphate 1-phosphatase | 0.000 | 0.001 | 5.6 |
| 4 | 4 | GREM1_Human | Gremlin-1 | 0.002 | 0.011 | 5.6 |
| 3 | 1 | HNRPK_Human | Heterogeneous nuclear ribonucleoprotein K | 0.000 | 0.001 | 5.5 |
| 1 | 1 | RAC1_Human | Ras-related C3 botulinum toxin substrate 1 | 0.000 | 0.001 | 5.4 |
| 1 | 1 | HXK1_Human | Hexokinase-1 | 0.000 | 0.001 | 5.3 |
| 1 | 1 | HEM6_Human | Oxygen-dependent coproporphyrinogen-III oxidase, mitochondrial | 0.000 | 0.001 | 5.2 |
| 10 | 9 | AEBP1_Human | Adipocyte enhancer-binding protein 1 | 0.007 | 0.039 | 5.2 |
| 2 | 1 | MOB1A_Human | MOB kinase activator 1A | 0.000 | 0.002 | 5.2 |
| 3 | 1 | CSRP2_Human | Cysteine and glycine-rich protein 2 | 0.000 | 0.001 | 4.8 |
| 3 | 2 | GRB2_Human | Growth factor receptor-bound protein 2 | 0.001 | 0.003 | 4.8 |
| 2 | 1 | KAD3_Human | GTP:AMP phosphotransferase AK3, mitochondrial {ECO:0000255\|HAMAP-Rule:MF_03169} | 0.000 | 0.001 | 4.7 |
| 4 | 2 | SSBP_Human | Single-stranded DNA-binding | 0.000 | 0.002 | 4.5 |

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|---|---|---|---|---|---|---|
| | | | protein, mitochondrial | | | |
| 2 | 1 | IF1AX_Human | Eukaryotic translation initiation factor 1A, X-chromosomal | 0.000 | 0.000 | 4.5 |
| 1 | 1 | PTPRK_Human | Receptor-type tyrosine-protein phosphatase kappa | 0.000 | 0.002 | 4.5 |
| 2 | 1 | SVEP1_Human | Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 | 0.000 | 0.001 | 4.4 |
| 1 | 1 | GSLG1_Human | Golgi apparatus protein 1 | 0.000 | 0.001 | 4.3 |
| 8 | 3 | SYWC_Human | Tryptophan--tRNA ligase, cytoplasmic | 0.001 | 0.006 | 4.2 |
| 2 | 2 | SPTB2_Human | Spectrin beta chain, non-erythrocytic 1 | 0.001 | 0.002 | 4.1 |
| 2 | 1 | PRIO_Human | Major prion protein | 0.001 | 0.003 | 4.1 |
| 6 | 1 | UB2L3_Human | Ubiquitin-conjugating enzyme E2 L3 | 0.000 | 0.001 | 4.0 |
| 1 | 1 | H31T_Human | Histone H3.1t | 0.001 | 0.002 | 4.0 |
| 5 | 2 | RNAS4_Human | Ribonuclease 4 | 0.001 | 0.005 | 3.9 |
| 2 | 1 | AHSA1_Human | Activator of 90 kDa heat shock protein ATPase homolog 1 | 0.001 | 0.003 | 3.8 |
| 2 | 1 | EIF3G_Human | Eukaryotic translation initiation factor 3 subunit G {ECO:0000255\|HAMAP-Rule:MF_03006} | 0.000 | 0.001 | 3.7 |
| 8 | 3 | RACK1_Human | Receptor of activated protein C kinase 1 | 0.003 | 0.009 | 3.4 |
| 1 | 1 | DUT_Human | Deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial | 0.001 | 0.002 | 3.3 |
| 3 | 1 | ANT3_Human | Antithrombin-III | 0.002 | 0.006 | 3.1 |
| 7 | 5 | DHE3_Human | Glutamate dehydrogenase 1, mitochondrial | 0.007 | 0.021 | 3.1 |
| 1 | 1 | PROS_Human | Vitamin K-dependent protein S | 0.000 | 0.001 | 3.0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 1 | NB5R2_Human | NADH-cytochrome b5 reductase 2 | 0.000 | 0.001 | 3.0 |
| 1 | 1 | HDGF_Human | Hepatoma-derived growth factor | 0.001 | 0.002 | 3.0 |
| 1 | 1 | PIN4_Human | Peptidyl-prolyl cis-trans isomerase NIMA-interacting 4 | 0.000 | 0.001 | 2.9 |
| 3 | 1 | TRFL_Human | Lactotransferrin | 0.010 | 0.028 | 2.9 |
| 3 | 1 | PPIL1_Human | Peptidyl-prolyl cis-trans isomerase-like 1 | 0.001 | 0.003 | 2.8 |
| 2 | 1 | HBA_Human | Hemoglobin subunit alpha | 0.002 | 0.005 | 2.8 |
| 3 | 2 | RBM3_Human | RNA-binding protein 3 | 0.001 | 0.004 | 2.7 |
| 1 | 1 | MSRA_Human | Mitochondrial peptide methionine sulfoxide reductase | 0.000 | 0.001 | 2.6 |
| 3 | 3 | FETUA_Human | Alpha-2-HS-glycoprotein | 0.007 | 0.017 | 2.3 |
| 4 | 4 | TRHDE_Human | Thyrotropin-releasing hormone-degrading ectoenzyme | 0.004 | 0.008 | 2.3 |
| 2 | 1 | VTDB_Human | Vitamin D-binding protein | 0.002 | 0.003 | 2.2 |
| 3 | 2 | SYNC_Human | Asparagine--tRNA ligase, cytoplasmic | 0.001 | 0.002 | 2.2 |
| 3 | 2 | FBLN3_Human | EGF-containing fibulin-like extracellular matrix protein 1 | 0.002 | 0.003 | 2.0 |
| 18 | 11 | HPLN1_Human | Hyaluronan and proteoglycan link protein 1 | 0.072 | 0.142 | 2.0 |
| 7 | 3 | A2MG_Human | Alpha-2-macroglobulin | 0.015 | 0.028 | 1.9 |
| 9 | 7 | SYHC_Human | Histidine--tRNA ligase, cytoplasmic | 0.011 | 0.020 | 1.8 |
| 1 | 1 | RUXG_Human | Small nuclear ribonucleoprotein G | 0.001 | 0.001 | 1.7 |
| 3 | 1 | SCOT1_Human | Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial | 0.000 | 0.001 | 1.7 |
| 4 | 1 | TRFE_Human | Serotransferrin | 0.000 | 0.001 | 1.6 |

# Chapter 5: General Discussion

# 5 General Discussion

Gliomas are the most malignant and aggressive form of brain tumors[1, 2]. Of these, Glioblastoma (GBM; WHO grade IV) is the most common and lethal subtype of glioma (55.1%)[3], and patients suffering from this tumor present a short overall survival after diagnosis. During the last decades, substantial advances have been made in the understanding of the origin, biology, genetics and clinical aspects of gliomas. However, the etiology of these tumors remains largely undetermined, and only moderate improvements in patients' clinical outcome were achieved. Also, despite a multimodal treatment, which includes surgical resection, radio- and chemo-therapy, these patients have a poorer prognosis with a median survival of 15 months[4]. Therefore, new and more efficient therapies are needed. Furthermore, gliomas are characterized as highly heterogeneous tumors, presenting a significant therapy resistance, which can be partly explained by the presence of a population of cells with stem cells characteristics, called glioma stem cells (GSCs). Taking this into account, it is clear that more studies are needed to better understand this devastating disease.

The work presented throughout this thesis focused in understanding glioma pathophysiology, with a special emphasis in GBM. Since single-variables studies are limited, we focused our studies on different intrinsic and extrinsic factors. For this, we studied multiple dimensions of glioma, from factors/events that may be causative, to those that influence their pathology, evolution, progression and aggressiveness, but always keeping their therapeutic importance in the loop. By using this approach, we contribute to the better understanding and management of these highly malignant tumors in the clinical setting.

We started by evaluating the genetic variants of two SNPs in the *TFG-β1* gene as molecular determinants involved on glioma etiology and prognosis. In the second work, we identified a new biomarker of GSCs identification, opening the possibility for further characterization of this malignant subpopulation. Finally, we studied the interplay between GBM cells and MSCs, a putative new therapeutic approach for cancer. Here, we evaluated the influence of MSCs' secretome on GBM behavior, and characterized the protein content of their secretome, identifying molecules that mediate the interaction between MSCs and GBM cells.

### 5.1 Relevance of *TGF-β1* genetic variants in glioma

Several studies have been exploring the role of genetic polymorphisms in the etiology, prognosis, and therapy response of human tumors[5]. It has been suggested that SNPs are the most frequent sources of human genetic variation, and that they can contribute to individual's susceptibility to cancer, including glioma[6]. SNPs in genes encoding proteins involved in the regulation of cancer hallmarks have been described as putative biomarkers for glioma susceptibility, including DNA repair, cell growth, apoptosis, cell cycle, proliferation, and metabolism[7-18]. Here, we examined for the first time in glioma the significance of two SNPs in the *TFG-β1* gene (-509C/T and 869T/C).

TGF-β1 is a member of the transforming growth factor beta superfamily of cytokines that is involved in the regulation of cellular proliferation, growth, apoptosis, differentiation, and immunity[19, 20]. This cytokine is a potent inhibitor of epithelial cells and astrocytes growth and proliferation, and the loss of this inhibition is associated with aggressive progression of cancer[21-23]. A hallmark feature of tumor cells is their acquired resistance to the inhibitory effect of TGF-β1 by mutations or inactivation of the TGF-β1 receptors (TGF-βRI and (TGF-βRII). In the late stages of cancer, TGF-β1 acts as an oncogenic factor stimulating angiogenesis, growth, invasion, proliferation, evasion to apoptosis, and decreases host immune response[24-26]. Moreover, it has been described that TGF-β1 plasma levels are increased and associate with cancer progression[27-33]. In glioma patients, a highly active TGF-β pathway confers poor prognosis and TGF-β1 acts as a proliferative and oncogenic factor[34, 35]. Due to its relevance in regulating cancer-related pathways, it is possible that SNPs in *TGF-β1* may contribute to individual's susceptibility to cancer, including glioma. Indeed, the genetic variants of the *TGF-β1* gene have been implicated in the susceptibility of a large range of tumors, including nasopharyngeal, esophageal, hepatocellular, lung, breast, gastric, head and neck, oropharyngeal, prostate, and liver cancers[31, 36-54]. However, until our study, none have reported the impact of *TGF-β1* SNPs on glioma susceptibility and prognosis.

Here, we performed a case-control study of cancer-free controls and glioma patients from a Portuguese population, and evaluated the putative impact of *TGF-β1* SNPs -509C/T and 869T/C variants in glioma risk and patient prognosis. Our results (chapter 2) show that the *TGF-β1* -509C/T and 869T/C polymorphism variants are not significantly associated with glioma susceptibility. These data is in line with previous studies in other tumor types reporting no associations between -509C/T polymorphism and increased risk of colorectal cancer, hepatocellular and nasopharyngeal carcinomas[55-59] or 869T/C and the risk to develop breast cancer and hepatocellular carcinoma[56, 60-67]. However, other works showed that these two polymorphisms were associated with the susceptibility to breast, gastric, ovarian, and prostate cancers[38, 68-70]. These inconsistent findings may result from studies with small sample sizes, different genetic and ethnic backgrounds of the studied populations, interactions with or contributions of other relevant etiologic factors that are virtually impossible to control for, disease heterogeneity, sample selection bias (of both controls and cases), small sample power, the use of different genotyping methods, and differences in the etiology and biology of different tumor types[71-75]. Aiming to better understand the real impact of *TGF-β1* SNPs in cancer susceptibility, recent meta-analyses showed that *TGF-β1* -509C/T variants increased the risk to certain cancer types, such as gastric, hepatocellular, and lung cancers[76-78], in specific populations. Particularly, it was demonstrated that TT genotype was significantly associated with susceptibility to gastric cancer in Asian population but not in Caucasians[78]. Interestingly, Li and colleagues have demonstrated that TT genotype from -509C/T and CC genotype from 869T/C polymorphisms were associated with the risk of asthma[79], results that fit well with our work (no association of TT or CC variants from -509C/T and 869T/C SNPs with glioma risk), since an inverse association between asthma and glioma has been reported in several case-control studies[80-83].

Very few robust prognostic and predictive markers are well established, which contributes for the devastating outcome of GBM patients[84]. Thus, the identification of other robust and clinically valuable molecular markers that associate with patient outcome is crucial. In our work, we found that GBM patients presenting the TT variant in -509C/T polymorphism and patients with CC genotype in 869T/C polymorphism presented longer OS, which indicates their potential as predictive markers of survival in GBM patients. This is in line with a previous work where it was shown that breast cancer patients carrying the CC genotype in 869T/C polymorphism presented longer overall survival[39]. This new finding requires further validation in independent studies, to confirm the clinical relevance of these SNPs in GBM patients.

A SNP located within the coding sequence of a gene may lead to an amino acid substitution, which in turn might alter the protein function, which could be functionally relevant and therefore be associated with susceptibility to cancer[85]. Considering that *TGF-β1* 869T/C polymorphism could lead to a leucine-to-proline substitution[78, 86], and that, in an *in vitro* study, it was shown that the C allele of the 869T/C cause an increase in TGF-β1 secretion compared with T allele[87], in the future it would be important to evaluate if the local secretion of TGF-β1 by glioma cells is also higher for CC homozygotes.

In future studies, and considering that the -509C/T polymorphism is located in the promoter region, it would be important to understand if the different genotypes influence the expression levels of *TGF-β1*. For that, mRNA extracted from glioma tumor tissues would be used to perform qRT-PCR analysis to evaluate the intratumoral levels of *TGF-β1*, and to correlate this expression with the different genotypes. Then, to establish a causal effect of the variant that affects *TGF-β1* expression, we could clone the promoter region of *TGF-β1* using a luciferase reporter vector. HEK293T cells (an easy-to-transfect cell line) should be transfected with two different vectors (with variant CC or TT), and the levels of luciferase measured. The levels of luciferase would be substitute for the levels of *TGF-β1* activation in both conditions. Additionally, it was described that *TGF-β1* -509C/T SNP influence the response to radio- and chemo-therapy of lung cancer patients[88]. Taking into consideration that glioma patients present high resistance to the current standard of care, it is possible that variants of *TGF-β1* -509C/T and 869T/C SNPs influence glioma patient response to treatment and should be clarified in future studies. Additionally, and considering that other functional SNPs of the *TGF-β1*, such as -800G/A (rs1800468) and 915G/C (rs1800471) were already associated with several cancer types susceptibility[67, 89], in the future, it would be important to evaluate if these SNPs are associated with glioma risk and prognosis. Moreover, GWAS, an approach that allows for a rapid whole genome sequencing and associates SNPs with disease[90], could be used to select and study other *TGF-ß1* polymorphisms that might be relevant in the context of glioma. Finally, future and independent studies are required to validate and expand our results to populations with different genetic backgrounds to better understand the broader importance of these SNPs in gliomas.

## 5.2 Is autofluorescence a valid and useful marker for Glioma Stem Cells identification and isolation?

In addition to an unclear etiology, malignant glioma is a dramatic disease as no curative therapies are available. GSCs are a subpopulation of cells that are involved in tumor initiation, progression and recurrence and evidences of their role in glioma pathophysiology continue to grow. GSCs are relatively undifferentiated cells presenting self-renewal ability that are recognized as a driving force supporting resistance to therapy, gliomagenesis, and aggressive recurrence[91]. Thus, it is imperative to efficiently identify and isolate GSCs for their better understanding and to find therapeutic targets capable of eliminating this highly malignant subpopulation.

So far, many characteristics and markers have been identified with varying degrees of specificity for GSCs and contributions to their phenotypes[91-93]. The most used method to isolate GSCs consists in sorting cells based on antibody recognition of specific cell membrane-bound proteins. Over the last years, several putative GSCs markers have been identified, including CD133, CD15, A2B5, among others (Table 5.1, reviewed in [94]). However, the use of these cell-surface markers has some limitations, since their expression is variable between gliomas, and they are also expressed in non-tumor stem cells[95]. It is likely that no marker will ever be uniformly informative for GSCs due to the inherent adaptability of cancer cells and because most tissue types contain multiple populations of stem cells expressing different markers[96]. Therefore, there is an unmet need to find more accurate and efficient isolation markers for GSCs identification and isolation. In this context, in the second work presented in this thesis (chapter 3) we investigated if an intrinsic autofluorescent phenotype was a biomarker of GSCs, as previously suggested for CSCs from carcinomas[97].

**Table 5.1:** Molecular markers associated with glioma stem cells (GSC)[94].

| Marker | Non-glioma cell types often associated with | Origin of GSCs (% of expression) | Refs |
|---|---|---|---|
| **A2B5** | OPCs | Adult GBM and anaplastic astrocytoma (33-90%). | 98, 99 |
| **Bmi1** | HSCs | Pediatric medulloblastoma, Anaplastic astrocytoma and GBM (n.a.). | 100 |
| **CD133** | HECs, and NSCs | Pediatric Medulloblastoma, Pilocytic and grade II astrocytomas, ependymoma and adult and pediatric GBMs (0.3-60%). | 101, 102 |
| **CD15** | Mouse ESCs | Adult medulloblastoma and GBM (0.7-70%) | 103, 104 |
| **CD44** | Mesenchymal cells | Adult GBM (17-79%). | 105 |
| **CD90** | BM-SCs and HSCs | Adult GBM (2-19%). | 106 |
| **Integrin 6α** | NSCs | Adult GBM (1-16%). | 107 |
| **KLF4** | ESCs and MSCs | Brain tumors (n.a.). | 108 |
| **L1CAM** | NPCs | Pediatric GBM and Adult gliomas (4-7%). | 109 |
| **Musashi** | Glial cells and NPCs | Adult Ependymoma, grade II oligodendroglioma and oligodendroastrocytoma, pilocytic astrocytomas, anaplastic astrocytomas and oligodendroglioma, and GBM (80%). | 100, 110 |
| **NANOG** | ESCs | Adult GBM (%). | 111 |
| **Nestin** | NSCs | Adult Ependymoma, grade II oligodendroglioma and oligodendroastrocytoma, pilocytic astrocytomas, anaplastic astrocytomas and oligodendroglioma, and GBM (96%). | 110, 112 |
| **Oct-4** | ESCs | Adult GBM (n.a.). | 111 |
| **Olig2** | OPCs and motor nuerons | Adult GBM (n.a.). | 113, 114 |
| **SOX2** | ESCs and neural tubes | Adult medulloblastoma, and GBM (46-95%). | 115-117 |

**Abbreviations:** OPCs, Oligodendrocyte progenitor cells; HSC, Hematopoetic stem cells; HECs, Hematopoetic, endothelial cells; NSCs, Neuronal stem cells; ESCs, Embryonic stem cells; BM-SCs, bone marrow-derived stem cells; MSCs, Mesenchymal stem cells; NPCs, Neural progenitor cells; n.a., not available.

In our work, we show that several human primary and established GBM cell lines present a proportion of Fluo$^+$ cells. These cells overexpressed GSCs markers (CD133, CD15, CXCR4, *Bmi1*, *Klf4*, *Nanog*, *Nestin*, *Oct3/4* and *SOX2*), presented an higher self-renewal ability, were resistant to therapy (treatment with TMZ and radiation led to an increased in the percentage of Fluo$^+$ cells), and were associated with shorter overall survival *in vivo*. Considering that GSCs are defined by functional characteristics including stem cell markers expression, therapy resistance, self-renewal capacity, differentiation into multiple lineages and tumor initiation and progression[96], all these results indicate that these Fluo$^+$ cells are GSCs. It is important to emphasize that although Fluo$^+$ cells have increased expression of a variety of CSCs makers, none of them was exclusively limited to this subpopulation, which contributes to the notion that these markers individually are not uniformly informative for CSCs.

Additionally, we show that the mechanism by which this GSCs are autofluorescent is due to the transport of RBF by the ABCG2 transporters into cytoplasmic vesicles. ABCG2, also known as BCRP belongs to one of the largest families of transporter proteins, the ABC transporters. ABC proteins can use the energy derived from ATP hydrolysis to perform a directed intermembrane movement of their substrates (primary active transporters), open or close a certain membrane channel (e.g. ion-channels) or regulate the permeability of multi-protein channel complexes (receptors)[118]. The overexpression of some members of the ABC transporters such as ABCG2, ABCB1, and ABCC1, is one of the major mechanisms responsible for multidrug resistance (MDR) phenotype. This phenotype is characterized by the ability that tumor cells have to display resistance to a wide range of drugs by performing the efflux of drugs across the cell membrane[118, 119]. ABC transporters are active in brain endothelial cells, contributing to the BBB, and playing a pivotal role in detoxification[120]. Particularly, ABCG2 is part of the protective mechanism that restricts entry of exogenous compounds, including small molecule chemotherapeutics into the brain. In fact, it was shown that some tyrosine kinase inhibitors (erlotinib and gefitinib) are substrates of ABCG2, which leads to very low brain concentrations of these drugs[121, 122].

Importantly, ABCG2 is expressed in normal stem cells, and plays an important role in promoting stem cell proliferation and the maintenance of the stem cell phenotype. However, this transporter is also expressed in a number of cancer cells and is a potential CSC marker, including in GSCs, being associated with tumorigenicity, proliferation, chemoresistance, and metastasis ability of cancer cells[123-126]. In GBM, it was demonstrated that CD133$^+$ cells highly express mRNA levels of *ABCG2* compared to CD133$^-$ cells[127]. Additionally, it was shown that ABCG2: i) has a molecular determinant role on the SP phenotype presented by GSCs, characterized by chemoresistance and tumorigenic properties[124]; ii) is a potential driver of glioma stemness by actively driving the expression of stem cell markers, and promotes GSCs self-renewal[128]; and iii) is associated with poor survival among GBM patients[129]. However, ABCG2 does not seem to affect either the response to radiation or tumor formation *in vivo*[128]. Importantly, the inhibition of this transporter with small molecule inhibitors result in reduced self-renewal of GBM neurospheres, suggesting, that ABCG2 is not just a marker of GSCs but also a promoter of. GSC self-renewa[129]. Taking into consideration all these previous studies, it will be important to evaluate the role of ABCG2 in our GBM Fluo$^+$ cells. In future studies we could silence *ABCG2* expression and evaluate if these Fluo$^+$ cells lose some of the GSCs characteristics, including stem cell markers expression and self-renewal ability of 3D neurospheres, as well as evaluate the *in vivo* tumorigenicity. Moreover, to evaluate if ABCG2 is an essential molecule in the stem phenotype, we should perform its overexpression in GBM Fluo$^-$ cells and observed if these cells become GSCs. Importantly, and using these GBM Fluo$^+$ cells silenced for *ABCG2* we should also confirm by flow cytometry analysis if a decrease in the autofluorescent content occurs. If these experiments reveal promising results, there are several compounds described to inhibit the action of ABCG2, including FTC, Ko-143, GF120918 (Elacridar), YHO-13177, YHO-1335, among others (reviewed in[130]) that could be used for further *in vitro* and *in vivo* experiments using either single agent or combination with TMZ strategies, in GBM Fluo$^+$ and Fluo$^-$ cells. However, it is important to notice that, to date, only few studies have investigated ABCG2 inhibitors clinical benefits in human trials and, none of these inhibitors are in clinical use.

Here, we show that the autofluorescent phenotype was due to an accumulation of RBF, a specific substrate for ABCG2 transporter[131, 132]. RBF is an essential vitamin that is present in food as free riboflavin or as the derivate flavin adenine dinucleotide (FAD) or flavin adenine mononucleotide (FMN). This vitamin is involved in numerous enzymatic reactions, in all forms of life, and performs key metabolic functions by mediating the transfer of electrons in biological oxidation-reduction reactions[133]. Additionally, RBF plays important roles in several cellular metabolic pathways, such as oxidative metabolism of fatty acids, amino acids and carbohydrates, and presents antioxidant properties[134, 135]. It is also involved in the metabolism of vitamin B6, folate, vitamin B12, and other vitamins; helps to maintain the integrity of the nervous system[136, 137]; is involved in the early postnatal development of the brain and gastrointestinal track[138-141], and is able to modulate carcinogen-induced DNA damage[142, 143] and inflammatory and immune responses[144, 145]. Some studies indicate that RBF deficiency increases the risk of cancer at certain sites whereas, others point to a possible attenuating effect of RBF in the presence of some carcinogens[143, 146]. Currently, besides ABCG2, other three RBF transporters have been characterized in humans: RFT1, RFT2, and RFT3[147-149]. RFT2 is mostly expressed in normal cells in the brain and is believed to play a key role in regulating brain RBF homeostasis[147]. Recently, it was demonstrated that RFT2 was overexpressed in glioma samples compared with normal brain, and was associated with WHO grade[150]. In addition, the silencing of *RFT2* was associated with glioma cell proliferation inhibition by promoting apoptosis and cell cycle arrest; a reduced invasion and migration; and a decreased tumor growth *in vivo*[150]. In fact, this same transporter was also described to be overexpressed in esophageal squamous cell carcinoma and involved in regulating cell cycle progression, cell proliferation, energy metabolism, tumorigenicity *in vivo*, and maintaining normal intracellular flavin status[151], Regarding, its expression in CSCs, including GSCs nothing is known. In our work, we see that FTC (inhibitor of ABCG2 transporting activity) treatment does not completely abrogate Fluo$^+$, and thus we hypothesize that the transport of RBF can be performed by other transporters, as RTF1, RFT2 and RTF3. In the future, the characterization of their expression, by Western blot, should be performed in FACS-sorted Fluo$^+$ and Fluo$^-$ GBM cells. If we observed that one or all of these transporters are involved in the uptake of RBF, similar studies as described above for ABCG2 inhibition, could be performed. Since, no inhibitors are described for these RBF transporters, we should modulate their expression in Fluo$^+$ and Fluo$^-$ GBM cells with alternative approaches as gene expression silencing. Additionally, future studies are needed to determine the exact role of RBF in GSCs. In fact, since this vitamin is involved in several redox reactions and has been described as presenting antioxidant properties, probably the presence of RBF on GSCs gives survival advantages to this highly malignant subpopulation. Thus, evaluating cell metabolism, as ROS production and mitochondrial respiration, of GSCs exposed to RBF or control conditions (no RBF) can help us to better understand its effects in GSCs.

It is of relevance that when we exposed our human GBM cell lines to basal medium (medium without riboflavin), we see that some cells remained autofluorescent. As cellular and tissue autofluorescence has been attributed to a spectrum of unrelated molecules such as vitamins (vitamin A, riboflavin, thiamine), structural proteins, porphyrins, lipofuscin, and ceroid pigment[152], it is reasonable to hypothesize that other fluorescent molecules can also partly contribute to the autofluorescent phenotype. One of the candidates is retinoic acid that presents a spectroscopic profile (330/500 nm) very similar to the profile of riboflavin (450/520 nm). Thus, in future experiments we should add retinoic acid to the culture media of GBM cells, and evaluate their level of autofluorescence.

Finally, and considering that GSCs share several characteristics with NSCs, it will be important to assess if this non-tumor stem cells also present an autofluorescent phenotype. Thus far, the autofluorescent phenotype has not been described in NSCs, however it was shown that these cells express ABCG2, even though in a small percentage comparing to GSCs[153]. In the future, the expression of ABCG2 and other RBF transporters should be evaluated in human NSCs cell lines, and in NSCs isolated from mice. Also, the autofluorescence content of NSCs should be assessed before and after RBF exposure, by flow cytometry analysis.

Taking into consideration all our results, it is clear that autofluorescence can be used as a marker to identify and isolate GSCs. This new GSC marker has a greater advantage comparing with those that are being used, such as CD133, CD15 (Table 5.1), since no extensive manipulation or antibodies are needed, eliminating any problem associated with epitope recognition.

In the future, it will be important to deeper characterize Fluo$^+$ and Fluo$^-$ GBM cells. Firstly, using FACS-sorted Fluo$^+$ and Fluo$^-$ GBM we could evaluate the signaling pathways that are altered in these subpopulations by performing phospho-arrays analysis. These results could tell us which pathways are differentially activated and which could hint for alternative therapeutic intervention to eliminate GSCs. Secondly, RNA sequencing could be performed to detect several differentially expressed genes. Those differentially up-regulated in the Fluo$^+$ subpopulation may constitute potential therapeutic targets for GSCs elimination. Thirdly, membrane proteomic analysis can also be performed in the two subpopulations, and in NSCs, to find new specific markers of the Fluo$^+$ GSCs subpopulation. This finding will constitute a great contribution to the field since, so far, no specific biomarker of GSCs exists, and could also represent potential therapeutic targets.

### 5.3 Can MSCs be safely used as a stem-cell based therapy for glioma treatment?

A relatively new and promising therapeutic approach to target malignant glioma is based on the use of MSCs. These cells have been explored as tolls for stem cell-based therapies since they present an intrinsic ability to migrate towards gliomas and can cross the BBB. However, the role that MSCs impose on glioma behavior is still controversial, and thus their validation as a safe therapeutic approach for glioma must be clarified. Some *in vitro* and *in vivo* studies demonstrated that MSCs present a tumor suppression function demonstrated by a tumor growth inhibition[154-160] while others showed that MSCs have a pro-tumoral function by stimulating tumor migration, invasion, and growth[161-165].

In order to clarify the influence of non-engineered MSCs on GBM behavior, in our last work (chapter 4) we evaluated the influence of HUCPVCs CM on GBM aggressiveness. One of the major mechanism by which MSCs impact cancer cells is by paracrine effects. Thus, to determine the effects of soluble factor released by MSCs on GBM cells we use CM from MSCs. The use of CM is advantageous in our setting due to its simplicity in allowing the detection and identification of any soluble factor-related effects in the culture media. Our data shows that GBM cells presented increased cellular viability, migration, proliferation and *in vivo* tumor growth when exposed to HUCPVCs CM. These results are in agreement with previous studies in different tumor types, including gliomas that demonstrated that MSCs may contribute to tumor growth/proliferation and migration[161, 165-172]. Several studies demonstrated that tumors have MSCs in their microenvironment, and these tumor associated-MSCs (TA-MSCs) enhanced tumor growth and invasiveness, and may contribute to the formation of distant metastases[162, 169, 173-176].

Particularly in gliomas, it has been shown that MSCs are recruited into these tumors where they play a functional role in the growth and aggressiveness of these tumors. Two independent studies demonstrated that cells similar to MSCs exist in glioma specimens[177, 178]. Later, Behnan and colleagues demonstrated that brain tumor-derived MSCs (BT-MSCs) increased GL261 cells' proliferation *in vitro*[172]. Similarly, Hossain *et al* were able to isolate cells presenting characteristics of MSCs, including surface antigens, classical MSC colony formation and mesenchymal differentiation potential, from fresh surgical glioma specimens, which they called glioma-associated human MSCs (GA-hMSCs)[163]. These GA-hMSCs presented similar functions as TA-MSCs in other cancer types by increasing the proliferation of GSCs, but were not tumorigenic. Moreover, they demonstrated that GA-hMSCs enhanced the stemness capacity of GSCs by both *in vitro* and *in vivo* assays[163]. Together, these studies indicate that TA-MSCs are present in glioma specimens, contributing to the aggressiveness of these tumors. Another study that demonstrated that caution must be considered in using MSCs as a new therapeutic approach was performed by Liu and colleagues, where they showed that rat BM-MSCs cultured with malignant rat glioma C6 cells without direct cell-cell contact became phenotypically malignant cells (decreased expression of p53 WT and increased expression of mutant p53 and mdm2, along with an aneuploid karyotype). Additionally, by an *in vivo* assay, these authors demonstrated that MSCs previously indirectly co-cultured with glioma C6 cells (when transplanted subcutaneously into immuno-deficient mice led to tumor development[179]. These results demonstrated that MSCs became malignant cancer cells when exposed to the tumor microenvironment and suggest that factors released from the cancer cells have a critical role in the malignant transformation of MSCs, highlighting the potential high risk of using MSCs as cell-based therapies for glioma treatment.

Taking into consideration that other studies demonstrated that MSCs do not promote glioma aggressiveness or present glioma-suppressing function[174, 180, 181], it is essential to standardize the methods used in different studies in order to more accurately understand if MSCs are definitely a valid and safe therapeutic approach to tackle cancer. For example, future studies should have into account tissue source and *in vitro* culture conditions of MSCs; type of tumor cells (primary and commercially available cell lines); variability of experimental methodologies concerning CM collection, and indirect or direct co-cultures; and studies using modified MSCs should include unmodified MSCs as control. In fact, it could be a better approach to use primary brain tumor in co-culture assays, as they may more accurately reflect the *in vivo* effects in future clinical applications. Moreover, since the major goal of these studies is their translation into the clinical setting, it is crucial that future studies use human MSCs and tumor cells, as we did in our work.

MSCs secrete a wide variety of biologically active molecules that modulate the activity of other cells. So far, several signaling molecules secreted by MSCs have been described to be involved in different biological processes such as ECM remodeling, angiogenesis, mitogenic remodeling, apoptosis inhibition, and tumor aggressiveness, among others (Figure 5.1)[182-184]. MSC-secreted proteins are capable of coordinating survival, migration, proliferation, and differentiation responses on healthy tissues and cancer cells through the activation of many signaling cascades. Additionally, the secretome of MSCs also presents proangiogenic effects, ECM components and proteins that regulate its composition. Finally, MSCs secrete a variety of chemoattractant molecules capable of recruiting diverse cell types, such as immune and progenitor cells (including MSCs themselves). Therefore, in order to clarify which proteins were mediating the response of GBM cells to HUCPVCs secretome, we performed proteomic analysis of HUCPVCs CM. We identified proteins that were significantly enriched in several pathways frequently altered in cancer (e.g. Wnt, PDGF and VEGF signaling pathways), in processes related to ECM organization and interaction, and in cellular adhesion and motion. Our results are in agreement with studies that evaluated the secretome of MSCs, since the proteins released by HUCPVCS that we identified were also described in other studies [185-187]. Particularly, proteins related with ECM and its organization (the most significantly represented molecular component and biological process), such as fibronectin, laminin, thrombospondin, metalloproteinases (MMP-1, MMP-2, MMP-3; MMP-14), and tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2) were found to be present in HUCPVCs secretome. Moreover, it has been described that MSCs secrete pro-angiogenic factors, such as VEGF [187], and once again in our study we observed that HUCPVCs CM present expression of VEGFC. Finally, several cytokines, growth factors and chemoattractant molecules, known to be secreted by MSCs, were also present in the secretome of HUCPVCs, including TGF-β1, HGF, TIMP-1 and -2, collagenases, IL-6, IL-F2, IL-F3, EGF, CCL2, and CXCL6, results that are in agreement with previous studies [185-188]. It is accepted that the pro-tumoral effects are mediated by secreted molecules and/or via direct cellular interactions [189-198]. For example, MSCs were shown to overexpress and secrete chemokine CCL5, which promoted breast cancer metastasis by stimulating chemokine CCR5 upregulation in breast cancer cells[199]. Furthermore, through upregulation and secretion of various cytokines (IL-6, IL-8), and chemokine CXCL12, TA-MSCs have been shown to promote tumor cell proliferation, metastatic spread, and resistance to chemotherapy in ovarian cancers[200].

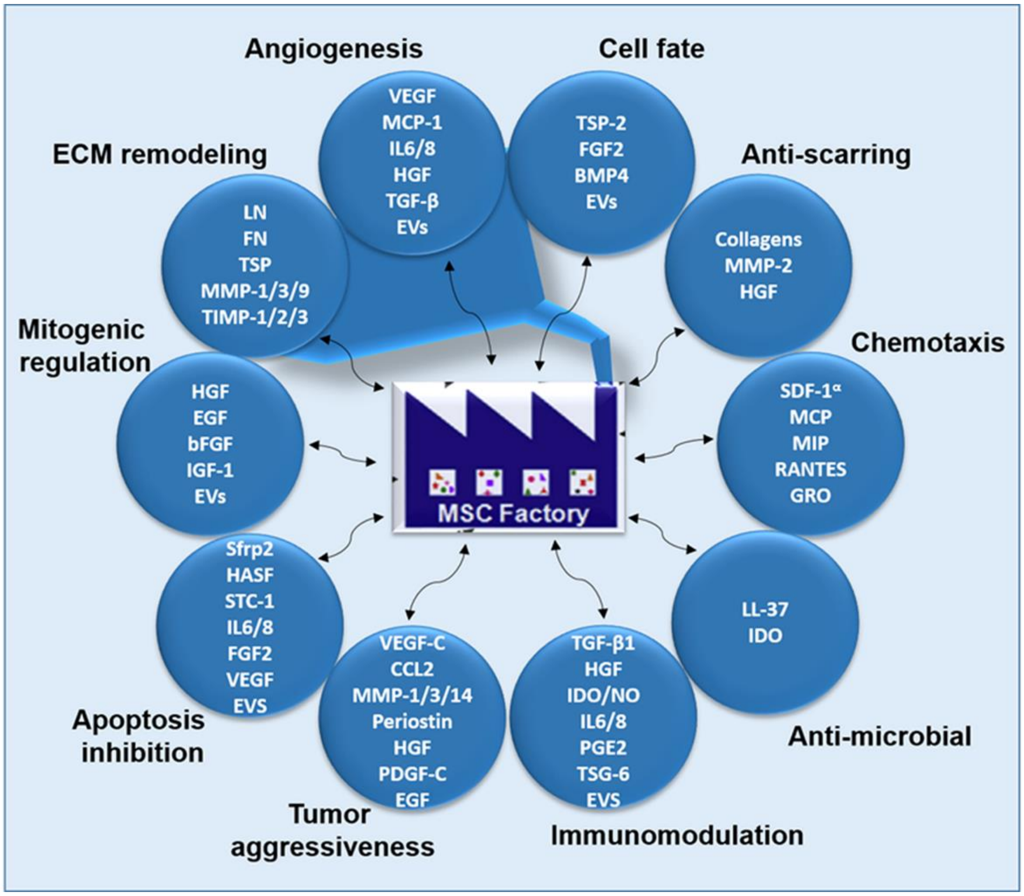

**Figure 5.1: Paracrine effects of MSCs.** MSC secretome functions include regulation of angiogenesis, apoptosis inhibition, cell differentiation, chemoattraction, ECM remodeling, immune response modulation, mitosis, antimicrobial properties, and tumor aggressiveness. Some of the main factors known to be secrete by cultured MSCs are indicated. MSCs respond to feedback signals coming from their target cells by modifying accordingly their "behavior" and secretome (Adapted from [201]).

All these results clearly demonstrated that MSCs secrete proteins that act on tumor cells, including GBM, raising concerns in using these stem cells as a therapeutic approach. In the future, and in order to better understand the crosstalk between MSCs and GBM cells, a direct (GBM cells and MSCs are cultured together) or indirect (communication through diffusible soluble factors without a direct cell-cell contact) co-culture system must be tested. In this way, we will be able to better evaluate how GBM cells modulate MSCs, as it allows both physical contact and soluble factor interactions, and how this modulation can influence back GBM cells. Additionally, it is important to evaluate other types of MSCs, such as BM-MSCs and ASCs, since a previous study performed by Akimotto *et al*, demonstrated that umbilical cord blood-derived MSCs (UCB-MSCs) inhibit, however ASCs promote, GBM proliferation[161]. In particular, if BM-MSCs prove promising they have many advantages to use in cell-based therapies compared to UCB-MSCs since they have a relatively large *ex vivo* expansion capacity, a low risk of viral infection, reduced donor morbidity and less-pronounced immunogenicity. In conclusion, for the clinical application of MSCs further investigations are necessary to establish effective and safety treatment strategies. These should have particular attention the source of the MSCs, the appropriate time intervals of administration and expression of tumor-supporting factors.

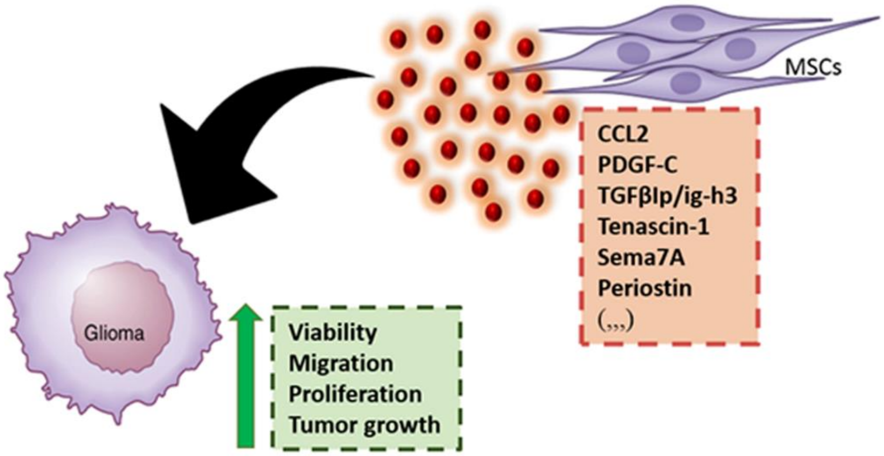

**Figure 5.2**: **Molecules secreted by MSCs enhanced aggressiveness features of GBM.** Proteins secreted by MSCs are related to cell adhesion and motion, ECM organization and interaction, and signaling pathways frequently altered in cancer, and are able to modulate GBM cells, by increasing tumor cell viability, proliferation, migration and, invasion.

### 5.4 Concluding remarks and future perspectives

Along this thesis, it has become clear that human gliomas are particularly dramatic diseases. Several discoveries are still to be made, especially regarding etiological and prognostic factors, as well as better therapeutic approaches. Here, we demonstrate for the first time that although *TGF-β1*-509C/T and 869T/C polymorphisms are not involved in glioma risk, they present a prognostic value in GBM patients (-509TT and 869CC genotypes are predictive markers of longer survival). Therefore, the study of these and other *TGF-β1* SNPs may prove relevant to the better understanding of the disease and for glioma patient prognostication. Since this is the first and only study on these SNPs in glioma, our data must be further validated. Moreover, we found a new and reliable marker for GSCs identification that is based on phenotypic characteristics of tumor cells, which can be used as a tool for a better understanding of this malignant subpopulation that does not require the use of antibodies, allowing to overcome problems associated with the use of cell surface markers. This straightforward and efficient isolation may be useful to better understand the biology of GSCs and identify additional biomarkers by characterizing their cell surface proteome. Finally, we give new insights regarding the use of MSCs for GBM treatment, demonstrating that caution should be taken of their use as clinical tolls for GBM treatment, and identify proteins released by MSCs that can promote GBM aggressiveness. Taken together, this work provides insights into some molecular and cellular players that regulate the pathophysiology of malignant gliomas, setting the stage for future works exploring therapeutic opportunities.

One molecule that is common between all the experimental works is TGF-β, which acts as an oncogenic factor in glioma, is overexpressed in GSCs, and plays a key role in MSCs recruitment to gliomas and to GSCs *in vivo*[195, 202, 203]. TGF-β induces the expression of many components of the ECM and cellular adhesion factors, and is involved in ECM remodeling through regulation of MMPs. Additionally, TGF-β acts in a paracrine fashion to regulate stromal cells, blood vessels, and local immune response. The net result of these interactions in malignant cancers is increase tumor cell invasion and angiogenesis. Regarding MSCs, it was shown that these cells express TGF-βRII on their surface. By using intracranial orthotopic human GSCs xenograft models, TGF-β1 was found to bind to both TGF-βRII and CD105, a coreceptor of TGF-βRII, and a canonical MSCs surface marker. The pro-inflammatory factor TGF-β1 is released by many gliomas, and interacts with TGF-βRII receptors located on the surface of MSCs. The presence of TGF-β1 was also correlated with the ability of MSCs to home to specific GSCs tumors [200, 202]. Importantly, it has been shown that the TGF-β/Smads signaling pathway induces immunosuppression by inhibiting NK cells, cytotoxic T lymphocytes, dendritic cells, and by upregulating T regulatory cells (reviewed in [204]), therefore, immunotherapeutic strategies to suppress TGF-β signaling may be promising for improving the prognosis of patients with malignant gliomas.

TGF-β plays a major role in glioma progression, in the future more studies are needed to better understand the molecular function associated with this cytokine. Using primary GBM cell lines that endogenously express TGF-β1 we could silence its expression by shRNA or more specifically perform TGF-β1 knockout using CRISPR-Cas9 technology, to evaluate both *in vitro* and *in vivo* glioma cells invasion, angiogenesis, immune responses, and interaction with cells present in the microenvironment. Finally, it would be important to better clarify if targeting TGF-β or its downstream signaling in combination with radio- and chemo-therapy could be a promising therapeutic approach in glioma patients. For that, compounds anti-TGF-β, such as AP12009, LY2157299, and GC1008, could be used. However, some caution to all anti-TGF-beta therapies is warranted as they may cause significant systemic side effects due to the critical role that TGF-beta plays in many normal physiological processes. Importantly, the combination of TGF-β signaling inhibitors with U.S. FDA-approved immune check-point blockade agents, such as anti-PD-L1, anti-CTLA4, and anti-PD1, antibodies, most likely would improve clinical outcomes over targeting a single pathway, especially as these antibodies have recently been shown to have efficacy in glioma models of glioma[205, 206].

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