



Neural Stem Cell Factors as Important Players in Glioblastoma Pathogenesis

Marta Moreno i Monasterio

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Neural Stem Cell Factors as Important Players in Glioblastoma Pathogenesis

Tesi presentada per
Marta Moreno i Monasterio

Tesi dirigida per la Dra. Núria de la Iglesia i Zaragoza

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Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)

Marta Moreno i Monasterio	Núria de la Iglesia i Zaragoza	Francesc Graus i Ribas
Doctoranda	Directora de la tesi	Tutor de la tesi

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Abbreviations

bFGF: basic Fibroblast Growth Factor
BFPP: Bilateral Frontoparietal Polymicrogyria
BM: Basement Membrane
BM: Bone Marrow
BMEC: Brain Microvascular Endothelial Cell
BMP: Bone Morphogenetic Protein
BV: Blood Vessel
CD44: Cluster of Differentiation 44
CHI3L1: Chitanae-3-like protein 1
CsA: Cyclosporin A
CTGF: Connective Tissue Growth Factor
CTRL: Control shRNA
DAPI: 4',6-diamidino-2-phenylindole
DIV: Days In Vitro
DFS: Disease Free Survival
DMSO: Dimethyl sulfoxide
ECM: Extracellular Matrix
EdU: 5-ethynyl-2'-deoxyuridine
EGF: Epidermal Growth Factor
EGFR: Epidermal Growth Factor Receptor
EMT: Epithelial to Mesenchymal Transition
ESC: Embryonic Stem Cell
FACS: Fluorescence Activated Cell Sorting

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FDR: False Discovery Rate
FK-506: Tacrolimus
FN1: Fibronectin 1
G-CIMP: Glioma CpG Island Methylator Phenotype
GBM: Glioblastoma
GFP: Green Fluorescent Protein
GIC: Glioma Initiating Cell
GPCR: G-protein coupled receptor
GPR56: G-protein coupled receptor 56
GPS: GPCR proteolytic site
GSEA: Gene Set Enrichment Analysis
Gy: Gray
HGF: Hepatocyte Growth Factor
HIF: Hypoxia Inducible Factor
HRE: Hypoxia Response Element
HSC: Hematopoietic Stem Cell
ICC: Immunocytochemistry
IHC: Immunohistochemistry
IKK: Inhibitor of Kappa Kinase
IL-6: Interleukin 6
IR: Ionizing Radiation
I κ B-SR: I κ B Super Repressor
I κ B: Inhibitor of Kappa B
KD: Knock Down
MCAM: Melanoma Cell Adhesion Molecule
MES: Mesenchymal
MET: Mesenchymal to Epithelial Transition
MMP: Matrix Metalloproteinases
MRI: Magnetic Resonance Imaging
mRNA: messenger RNA
MSC: Mesenchymal Stem Cell

NEMO: NF- κ B Essential Modulator

NES: Normalized Enrichment Score

NF- κ B: Nuclear Factor- κ B

NFAT: Nuclear Factor Activated T cells

NSC: Neural Stem Cell

OLIG2: Oligodendrocyte transcription factor 2

O/N: Overnight

OPC: Oligodendrocyte Precursor Cell

OS: Overall Survival

PBS: Phosphate-Buffered Saline

PCA: Principal Component Analysis

PFA: Paraformaldehyde

PN: Proneural

RNA: Ribonucleic acid

RT-qPCR: Reverse Transcription quantitative Polymerase Chain Reaction

S3C: Constitutively active STAT3

SCC: Spearman's rank Correlation Coefficient

SEM: Standard Error of the Mean

SERPINE1: Endothelial Plasminogen Activator Inhibitor

SGZ: Subgranular Zone

SH5: GPR56 shRNA #5

shRNA: short hairpin RNA

siRNA: small interfering RNA

SNAI1-2: Snail Family Zinc Finger 1-2

SOX2: Sex determining region-box 2

SOX9: Sex determining region-box 9

SP: Side Population

STAT3: Signal Transducer and Activator of Transcription 3

SVZ: Subventricular Zone

TAZ: Tafazzin

TCGA: The Cancer Genome Atlas

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TF: Transcription Factor
TFT: Transcription Factor Target gene sets
TGFB1: Transforming Growth Factor b1
TGF- β : Tumor Growth Factor β
TGM2: Transglutaminase 2
TMZ: Temozolamide
TIMP1: Metallopeptidase Inhibitor 1
TNF- α : Tumor Necrosis Factor α
U-p65: Unphosphorylated p65
U-STAT3: Unphosphorylated STAT3
WB: Western Blot
ZEB1: Zinc Finger E-box Binding Homeobox 1

Introduction

1.1 Characteristics of gliomas

1.1.1 Definition and classification

Over 11 million individuals are diagnosed with cancer annually and it is estimated that the number of new cases will increase in future years. Malignant gliomas are the most common type of primary brain tumor with an incidence of 5/100,000 individuals (Bhatt et al., 2010). Although their incidence is not as high as with other tumors, their anatomical localization and aggressiveness converts these malignancies into a huge burden on society.

Traditionally, gliomas are defined as tumors that phenotypically resemble glial cells, such as astrocytes, oligodendrocytes or ependymal cells. Accordingly, gliomas are divided into astrocytomas, oligodendrogliomas, ependymomas and mixed gliomas (oligo-astrocytomas). Astrocytomas are the most common type of gliomas in both adults and children. The World Health Organization (WHO) grades astrocytomas based on histologic features according to their degree of malignancy. Astrocytomas are subdivided into four grades with an increasing degree of malignancy: low-grades, including grade I (or pilocytic astrocytoma) and grade II (or diffuse astrocytoma), and high-grades, including grade III (or anaplastic astrocytoma) and grade IV (or glioblastoma multiforme, GBM) (Sulman et al., 2009).

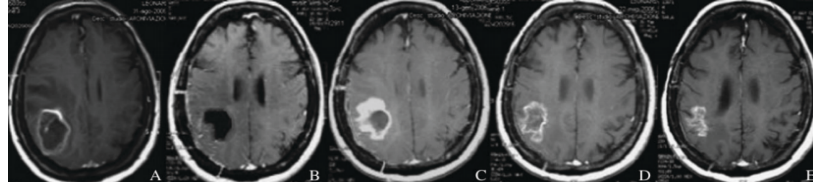


Figure 1.1: *Clinical course of a 65-year-old patient with glioblastoma.*

(A) Presurgical magnetic resonance imaging (MRI) scan. (B) Postsurgical MRI scan. (C) MRI scan performed one month after combined temozolomide plus radiotherapy (TMZ/RT). Treatment with adjuvant TMZ was continued. (D) Four months later, MRI scan was performed during administration of maintenance TMZ. (E) MRI scan eight months later during administration of maintenance TMZ. Figure reproduced from Stupp et al., 2008.

In this PhD thesis, we have focused on the study of GBM, the most aggressive and common malignant primary brain tumor, accounting for 60-70% of all gliomas (Goodenberger and Jenkins, 2012). The median survival time of GBM is only of 12-15 months and despite huge efforts to improve disease outcome mostly all GBM patients succumb to the disease within less than 3 years (Figure 1.1) (Furnari et al., 2007; Jovčevska et al., 2013). Consequently, GBM still remains as one of the most aggressive and clinically challenging human tumors.

1.1.2 GBM epidemiology

Glioblastoma appears mostly in adults above the age of 55 (87%). This tumor is more common among men compared to women (3:2) and less common among African or Asian descent compared to Caucasian population. Genetic predisposition has been described in 5-10% of patients and some low-penetrance susceptibility alleles have been identified (Wrensch et al., 2009; Shete et al., 2009). Furthermore, patients with rare genetic tumor syndromes such as Li Fraumeni syndrome, Turcot's syndrome or neurofibromatosis 1 and 2 have a higher risk to develop GBM (Oliver et al., 2009). Besides previous exposure to ionizing radiation (IR), no other predisposition factor has been identified (Dunn et al., 2012).

According to their clinical presentation, GBM are divided into primary or secondary. The most common are primary GBM, which arise *de novo* without prior clinical history of glioma, usually in patients over 50 years. Conversely, secondary GBM tend to arise from lower-grade glioma lesions, usually in younger

patients (below 45 years). However, primary and secondary GBM are morphologically and clinically indistinguishable (Sulman et al., 2009).

1.1.3 GBM pathological features and therapy

The hallmark features of diffuse infiltration, high proliferative rate, necrosis, angiogenesis, resistance to apoptosis and genomic instability define GBM (Kotliarova and Fine, 2012). Classically, GBM were named “multiforme” due to their high degree of heterogeneity either on the cytopathological and the genomic levels. Altogether, these malignant features make GBM one of the most difficult cancers to understand and treat. Surgical resection followed by radiotherapy with adjuvant or maintenance chemotherapy is the current standard therapy for newly diagnosed patients (Stupp regimen) (Stupp et al., 2005). Temozolamide (TMZ) is the oral alkylating agent wider used in GBM chemotherapy due to its effectiveness in crossing the blood-brain barrier. A Food Drug Administration (FDA)-approved anti-VEGF humanized monoclonal antibody that interacts with VEGF receptor of tumor endothelial cells (Bevacizumab) has improved progression-free survival but not the overall survival. However, appearance of resistance and increased aggressiveness has been associated to this treatment (Hinnen and Eskens, 2007).

Despite treatment, tumor recurrence appears in almost all patients. Consequently, several new targeted agents and novel approaches are being developed. Targeted therapies such as EGFR tyrosine kinase inhibitors (erlotinib and gefitinib), mTOR antagonists (temsirolimus and everolimus), PDGFR inhibitors (imatinib) or inhibitors of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin (cilengitide) have shown minimal benefit until now (Tanaka et al., 2013). On the other hand, vaccines targeting tumor-specific epitope (like EGFR variant III), radiotherapy with intra-operative injection of herpes simplex virus-thymidine kinase gene vectors (Cerepro) or radiolabeled monoclonal antibodies against tumor-specific antigens (like EGFR, tenascin or integrins) have shown some efficacy in clinical and preclinical trials (Table 1.1) (Daga et al., 2011; Sampson et al., 2009; Li et al., 2010; Veeravagu et al., 2008). Despite new insights into the molecular landscape of GBM in the last decade, the identification of robust prognostic factors is still required for a better understanding of gliomagenesis and the improvement of current therapies.

Table 2 Molecular-targeted therapies in clinical trials		
Agent	Target	Selected reference/trial identifier
EGFR-targeted therapies		
Erlotinib	EGFR	Brown <i>et al.</i> (2008), ⁵⁷ van den Bent <i>et al.</i> (2009) ⁶⁰
Gefitinib	EGFR	Franceschi <i>et al.</i> (2007), ¹⁷³ Kreisl <i>et al.</i> (2009) ⁶⁹
Cetuximab	EGFR	Neyns <i>et al.</i> (2009) ⁶²
Lapatinib	EGFR, HER2	Thiessen <i>et al.</i> (2010) ⁶³
Afatinib	EGFR	NCT00977431
Dacomitinib	EGFR	NCT01112527, NCT01520870
Nimotuzumab	EGFR	NCT00753246
PI3K/Akt/mTOR-targeted therapies		
Temsirolimus	mTOR	Galanis <i>et al.</i> (2005) ⁶⁸
Everolimus	mTOR	Kreisl <i>et al.</i> (2009) ⁶⁹
BKM120	PI3K	NCT01339052, NCT01349660, NCT01473901
XL765	PI3K, mTOR	NCT00704080
Others		
Sorafenib	PDGFR- α , β , VEGFR-2,3, BRAF, c-Kit, Ras	Hainsworth <i>et al.</i> (2010), ¹⁰⁸ Reardon <i>et al.</i> (2011), ¹⁰⁹ NCT01434602, NCT00329719, NCT00734526, NCT00884416
Imatinib	PDGFR- α , β , c-Kit, Bcr-Abl	Wen <i>et al.</i> (2006), ⁷⁴ Reardon <i>et al.</i> (2009), ⁷⁵ Dresemann <i>et al.</i> (2010) ⁷⁶
Tandutinib	PDGFR- α , β , c-Kit, Flt3	NCT00379080, NCT00667394
Dasatinib	PDGFR- α , β , Src, Bcr-Abl, c-Kit, EphA2	NCT00423735, NCT00869401, NCT00892177, NCT00895960
PD-0332991	CDK4, CDK6	NCT01227434
Vorinostat	Histone deacetylase	Galanis <i>et al.</i> (2009), ⁸¹ Friday <i>et al.</i> (2012) ⁸²
Panobinostat	Histone deacetylase	NCT00859222
Valproic acid	Histone deacetylase	NCT00302159
Bortezomib	Proteasome	Friday <i>et al.</i> (2012), ⁸² Phuphanich <i>et al.</i> (2010) ⁸³
Iniparib	Poly(ADP-ribose) polymerase	NCT00687765
GSC-targeted therapies		
RO4929097	γ -secretase	NCT01119599, NCT01122901
Vismodegib	Smoothed homolog	NCT00980343
Antiangiogenic therapies		
Bevacizumab	VEGF-A	Friedman <i>et al.</i> (2009), ⁹⁶ Lai <i>et al.</i> (2011) ⁹⁸
Aflibercept	VEGF-A, VEGF-B, PlGF	de Groot <i>et al.</i> (2011) ¹⁰²
Cediranib	VEGFR-1,2,3, PDGFR- α , β , FGFR-1, c-Kit	Batchelor <i>et al.</i> (2007) ¹⁰³
Sorafenib	VEGFR-2,3, PDGFR- α , β , BRAF, c-Kit, Ras	Hainsworth <i>et al.</i> (2010), ¹⁰⁹ Reardon <i>et al.</i> (2011) ¹¹⁰
Sunitinib	VEGFR-2, PDGFR- β , c-Kit, RET, Flt3	Neyns <i>et al.</i> (2011) ¹¹⁶
Vandetanib	VEGFR-2, EGFR, RET	Drappatz <i>et al.</i> (2010) ¹¹⁷
Cabozantinib	VEGFR-2, Met, RET, c-Kit, Flt3, Tie-2	Wen <i>et al.</i> (2010) ¹¹⁵
Cilengitide	$\alpha v\beta 3$, $\alpha v\beta 5$ integrins	Reardon <i>et al.</i> (2008), ¹²¹ Stupp <i>et al.</i> (2010) ¹²²
Enzastaurin	PKC- β , Akt	Kreisl <i>et al.</i> (2010) ¹¹⁹
AMG386	Ang-1, Ang-2	NCT01290263, NCT01609790
Abbreviations: FGFR, fibroblast growth factor receptor; GSC, glioma stem-like cell; mTOR, mammalian target of rapamycin; PlGF, placental growth factor; PDGFR, platelet-derived growth factor receptor.		

Table 1.1: *Molecular-targeted therapies in GBM clinical trials.*

List of the different compounds that are being tested for GBM treatment and their molecular targets. Figure adapted from Tanaka *et al.*, 2013.

1.2 GBM heterogeneity

GBM are highly heterogeneous tumors, hence the term glioblastoma multiforme, which was classically used to name this aggressive human malignancy. In this section, the different evidences and theories that try to explain GBM heterogeneity are reviewed.

1.2.1 Inter-tumoral heterogeneity: Molecular classification

Despite all GBM having similar anatomopathologic characteristics, these tumors are very different at the molecular level from one patient to another. According to their gene expression profile, The Cancer Genome Atlas (TCGA) Research Network has classified GBM in four different molecular subtypes: Proneural, Neural, Classical and Mesenchymal (Figure 1.2) (Verhaak et al., 2010). The Classical subtype is characterized by chromosome 7 amplification, chromosome 10 loss, *EGFR* amplification or mutation, loss of *TP53* and *RB* alterations. Neural stem cell (NSC) markers such as Nestin (*NES*), and members of the Notch (*NOTCH3*, *JAG1* and *LFNG*) and Sonic hedgehog (*SMO*, *GAS1* and *GLI2*) pathways are highly enriched in this GBM subtype. Conversely, the Neural subtype displays high expression of neural markers, such as *NEFL*, *GABRA1*, *SYT1*, and *SLC12A5*. In this GBM subtype, genetic alterations are very similar to the classical subtype with high frequency of *TP53* mutations and *EGFR* amplifications. The Proneural subtype (PN) is characterized by *PDGF* alterations, *IDH1* and *IDH2* mutations and *TP53* and *CDKN2A* loss. These tumors present high expression of oligodendrocytic markers such as *PDGFRA*, *NKX2-2* and *OLIG2*. Finally, the Mesenchymal subtype (MES) is characterized by *NF1* and *PTEN* alterations. This subtype displays expression of both mesenchymal markers, such as *CHI3L1* (also known as *YKL40*) and *MET*, as well as astrocytic markers, such as *CD44* and *MERTK*. Furthermore, this subtype also shows hyperactivation of NF- κ B and TNF- α signaling pathways. Recent studies have revealed that six master transcription factors (TF) control the transcriptional network of the mesenchymal subtype: C/EBP β , STAT3, RUNX1, bHLHB2, FOSL2 and ZNF238 (Carro et al., 2010).

The prognostic value of the molecular subclassification of GBM is still under review. Some authors have observed that a gene signature associated with

neurogenesis (PN subtype) generally has improved survival compared with the MES subtype (Phillips et al., 2006). On the contrary, other studies have not found a significantly improved outcome for patients with a PN-subtype GBM (Verhaak et al., 2010). Otherwise, alterations related to PN GBM such as *IDH1* mutations have been associated with an increase in the overall survival of GBM patients (Parsons et al., 2008; Sanson et al., 2009; Yan et al., 2009), whereas elevated expression of mesenchymal genes, such as *CD44* or *TGF- β* is associated to poorer patient prognosis (Figure 1.3) (Pietras et al., 2014; Bruna et al., 2007).

Epigenetic analysis of TCGA samples further revealed a distinct molecular subgroup in human GBM. The glioma CpG Island Methylator Phenotype (G-CIMP) is strongly associated with a favorable prognosis in GBM. G-CIMP-positive patients represent a subset of PN-subtype GBM tightly associated with *IDH1* mutations and secondary tumors (Noushmehr et al., 2010). Although the transcriptional profile for some G-CIMP-positive tumors resembles those of other PN GBM (non-G-CIMP), their biology is significantly different in that the G-CIMP-positive display distinct characteristics, such as copy number alterations and prolonged patient survival. These data suggest that these tumors represent higher-grade versions of grade II and III gliomas, which are also commonly G-CIMP-positive. (Ohgaki and Kleihues, 2013; Noushmehr et al., 2010). The molecular features within G-CIMP-positive GBM include the silencing of a large subset of genes involved in specific functional categories such as markers of mesenchyme, tumor invasion and extracellular matrix (Noushmehr et al., 2010), supporting the idea that a mesenchymal genes signature display a poor patient prognosis (Phillips et al., 2006). Despite the important advances on GBM classification of the recent years, further characterization of molecular subsets of GBM is required to provide new insights into the initiation and progression of GBM and to eventually develop targeted drug treatments for GBM patients.

1.2.2 Intra-tumoral heterogeneity: Glioma Initiating Cells

In addition to the inter-tumoral heterogeneity, different subpopulations of cells in the tumor bulk have been described in GBM. Two different hypothesis have been postulated to explain this intra-tumoral heterogeneity: the stochastic model or clonal evolution model and the hierarchical model or cancer stem cell model (Figure 1.4). In the clonal evolution model, tumors are proposed to arise from normal cells that undergo somatic mutations which make them be-

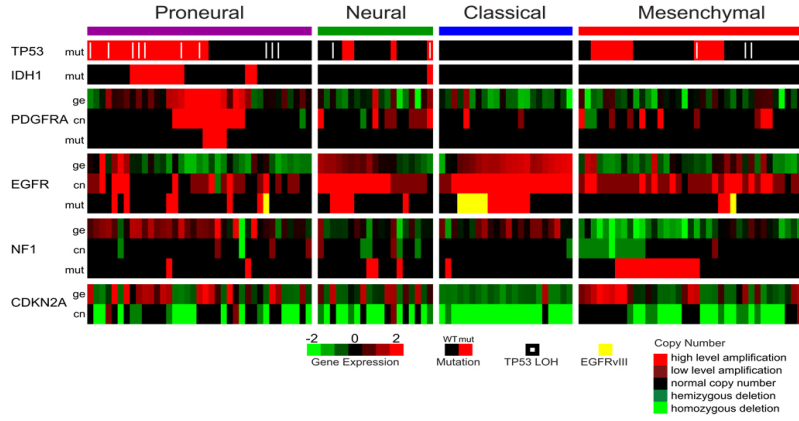


Figure 1.2: *Integrated view of gene expression and genomic alterations across GBM subtypes.*

Main GBM subtypes according to its copy number and gene expression. Figure reproduced from Verhaak et al., 2010.

come tumorigenic. These tumor cells generate offspring which at some point can accumulate further mutations, thereby creating a tumor with phenotypically different cancer cells, all of them with similar tumorigenic potential. On the other hand, the cancer stem cell model hypothesizes that tumor initiation and propagation are driven by a small subpopulation of cells with stem-like properties, also known as cancer initiating cells (CIC). In analogy to normal stem cells, these stem-like cells undergo multilineage differentiation driven by epigenetic changes, thereby generating phenotypically diverse non-tumorigenic cancer cells that form the tumor bulk. In addition to explaining intra-tumoral heterogeneity, this model also explains the epigenetic differences between tumorigenic and non-tumorigenic cells found within a tumor. Therefore, identifying the stem-like population within a tumor is of crucial importance to understand whether tumors follow the cancer stem cell model.

In GBM, several groups have described the presence of stem-like cells known as glioma-initiating cells (GIC). GIC are considered to be tumor drivers, responsible for tumor initiation and resistance to therapeutic agents (Reya et al., 2001; Fomchenko and Holland, 2005; Sanai et al., 2005; Kim and Dirks, 2008; Natsume et al., 2011; Piccirillo et al., 2009; Woolard and Fine, 2009; Sulman et al., 2008). There have been several attempts to phenotypically define the

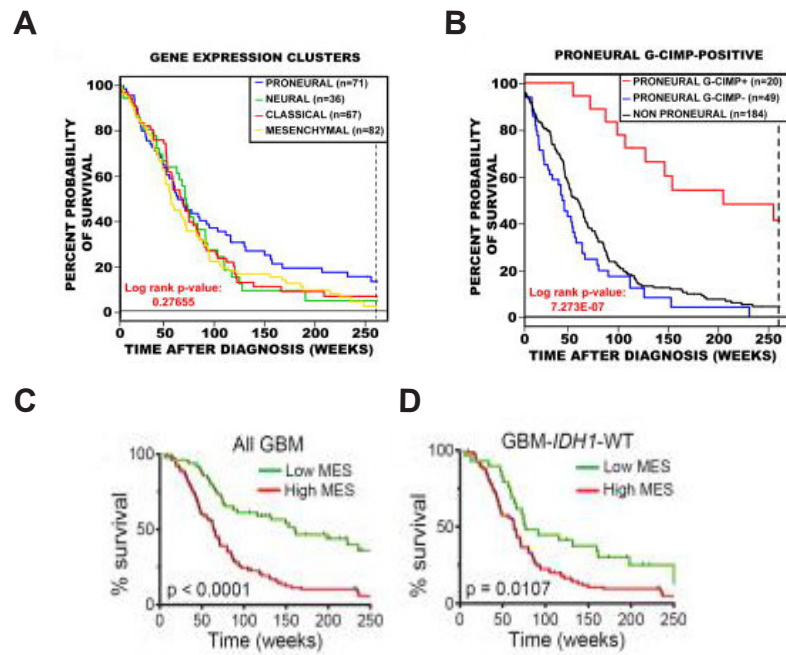


Figure 1.3: *Prognostic value of the molecular classification of GBM.*

(A) Kaplan-Meier survival curves comparing the four GBM gene expression subtypes. (B) Kaplan-Meier survival curves comparing PN G-CIMP-positive, PN G-CIMP-negative and all non-proneural GBM tumors. (C) Kaplan-Meier survival of GBM patients based on PN/MES metagene scores. (D) Kaplan-Meier curves showing survival of *IDH* wild-type GBM patients based on PN/MES metagene score. Log rank test was used to assess statistical significance. Figures reproduced from Bhat et al., 2013; Noushmehr et al., 2010.

GIC population. CD133 has been extensively used as a GIC marker since the CD133⁺ brain tumor fraction contains cells with stem-like properties that are capable of tumor initiation in mouse models (Singh et al., 2004). However, latter studies demonstrated that some CD133⁻ cancer cells also have stem-like properties as well as tumor propagating potential (Singh et al., 2003; Son et al., 2009; Chen et al., 2010). Other authors postulate that GIC can be identified by the expression of the ABC transporter and characterized by Hoechst dye exclusion, known as Side Population (SP) (Buijs et al., 2012). More recently, other groups have pointed to CD44 as a putative GIC marker, focusing on the acquisition of mesenchymal features by GIC, which might confer poor patient prognosis (Wang and Bourguignon, 2011; Anido et al., 2010).

Recent studies show that tumor heterogeneity is a dynamic process that changes with time. GBM contain multiple cellular states with the distinct transcriptional programs of the different molecular subtypes (Patel et al., 2014). Within the same tumor, cells with PN features coexist with MES-like cells that express different stem-like markers. Additionally, spontaneous conversion of PN to MES tumors has been observed (Bhat et al., 2013; Sottoriva et al., 2013). Furthermore, some authors suggest that MES-subtype GBM evolve from PN-like lesions. These recent data connect the different GBM subtypes in an evolutionary framework where the primordial tumors are PN in character, with the other subgroups evolving from them (Ozawa et al., 2014). Given the diverse number of histological subtypes, molecular subtypes and the increasing number of phenotypically different stem-like cells, further studies facing the molecular mechanisms that drive this heterogeneity are urgently required.

1.3 GIC and NSC

NSC and neural progenitor cells have a unique expression pattern of cell surface markers, cytoskeletal proteins, transcription factors and growth factors that are largely shared with GIC. GIC express NSC developmental genes which contribute to tumor proliferation and self-renewal. During development, Sonic Hedgehog (SHH) is a pivotal regulator of patterning and proliferation in the cerebellum (Dahmane and Ruiz i Altaba, 1999). Analogously to NSC, SHH signaling is also implicated in the pathogenesis of GBM (Gruber Filbin et al., 2013). Other important factors in NSC, such as NOTCH and WNT are also

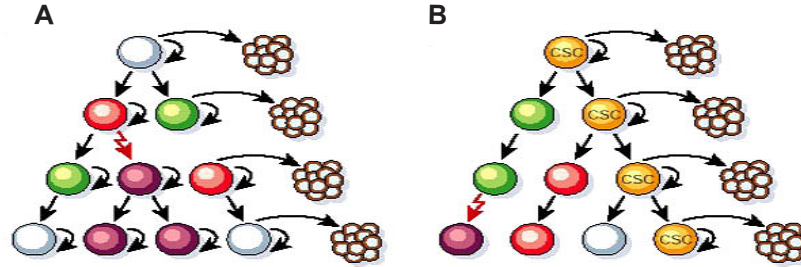


Figure 1.4: *Two different models have been proposed to explain tumor heterogeneity.* The stochastic model (A) proposes that heterogeneity is achieved by random mutations in tumor cells giving rise to different tumorigenic clones, all of which can function as tumor-founding cells. Conversely, the hierarchical model (B) implies that only a small subpopulation of tumor stem-like cells can proliferate extensively and sustain the growth and progression of a neoplastic clone. In this model, tumor bulk cancer cells are the progeny of these rare tumor stem cells. Figures reproduced from Vescovi et al., 2006.

involved in the proliferation and survival of GIC (Noushmehr et al., 2010; Bruna et al., 2007).

The tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (*PTEN*) is involved in the control of stem cell proliferation, differentiation and migration (Li et al., 2003). Deletion of *PTEN* in NSC leads to persistent self-renewal and expansion accompanied with a decrease in apoptosis (Gregorian et al., 2009; Groszer et al., 2001). Similarly, deletion of *PTEN* in GBM increases the size of the Side Population and the expression and the activity of the ABCG2 transporter (Bleau et al., 2009). Interestingly, *PTEN* mutations and loss of heterozygosity are frequently described in GBM. In addition, the cytoskeletal protein nestin, a type IV intermediate filament which is expressed by neural progenitors in the embryo and in the adult, is also expressed in human GBM (Wiese et al., 2004; Yang et al., 2008b).

Growth factors, such as the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) have a major role on NSC biology, as well as on gliomagenesis (Jackson et al., 2006; Doetsch et al., 2002; Holland et al., 2000). In GBM, *EGFR* amplifications or expression of *EGFRvIII*, a constitutively active mutant, have been found in nearly half of all GBM. The transforming growth factor β (TGF- β) is also required for the maintenance of GIC and NSC properties (Bonnert et al., 2006; Peñuelas et al., 2009; Watabe and Miyazono, 2009;

Joo et al., 2012). Finally, bone morphogenetic proteins (BMP), which have a pivotal role in normal NSC differentiation, trigger the differentiation of GIC dramatically reducing their tumor propagating abilities (Piccirillo and Vescovi, 2006; Bond et al., 2012).

Normal NSC reside in a unique microenvironment or niche. In the adult, NSC and lineage-restricted progenitor cells have been described in several regions of the adult mammalian brain including the subventricular zone (SVZ) of the lateral ventricles, the subgranular zone (SGZ) of the hippocampal gyrus, striatum, frontal and temporal cortex, as well as subcortical white matter (Figure 1.5) (Alvarez-Buylla et al., 2002). The SVZ is the largest reservoir of these cells in the human brain. Within the SVZ, NSC lie in close apposition to endothelial cells in blood vessels, forming a perivascular niche (Palmer et al., 2000). Similarly, GBM are highly vascularized tumors and GIC are found closely associated with tumor capillaries (Calabrese et al., 2007).

Due to the similarities between NSC and GIC, numerous studies have focused on the study of NSC factors and properties in the context of gliomas, in order to better understand GIC properties. However, other developmental programs have been found in GBM. An embryonic stem cell-like gene expression signature has been associated with poorly differentiated human tumors, including GBM (Ben-Porath et al., 2008). Additionally, other transcriptional pathways active in non-neural lineage cells have been also found in GBM (Verhaak et al., 2010).

1.4 Cell-of-origin

As described above, a great deal of experimental data suggests that the cell capable of initiating and propagating GBM has properties of NSC. Therefore, it is plausible to think that the initial cell that acquires tumorigenic capabilities and originates the tumor might be a normal NSC. However, other cell types might also accumulate mutations that might make them acquire stem-like properties and render them tumorigenic. The true nature of the cell of origin of GBM has been an issue of discussion for decades and it's still an open debate.

Several genetic studies support the NSC origin of gliomas. GBM may arise from normal NSC or progenitors within the brain, since diverse glioma-relevant mutations targeting NSC *in vivo* generate glioma-like lesions with high fidelity (Alcantara Llaguno et al., 2009; Zhu et al., 2005; Wang et al., 2009b). Other stud-

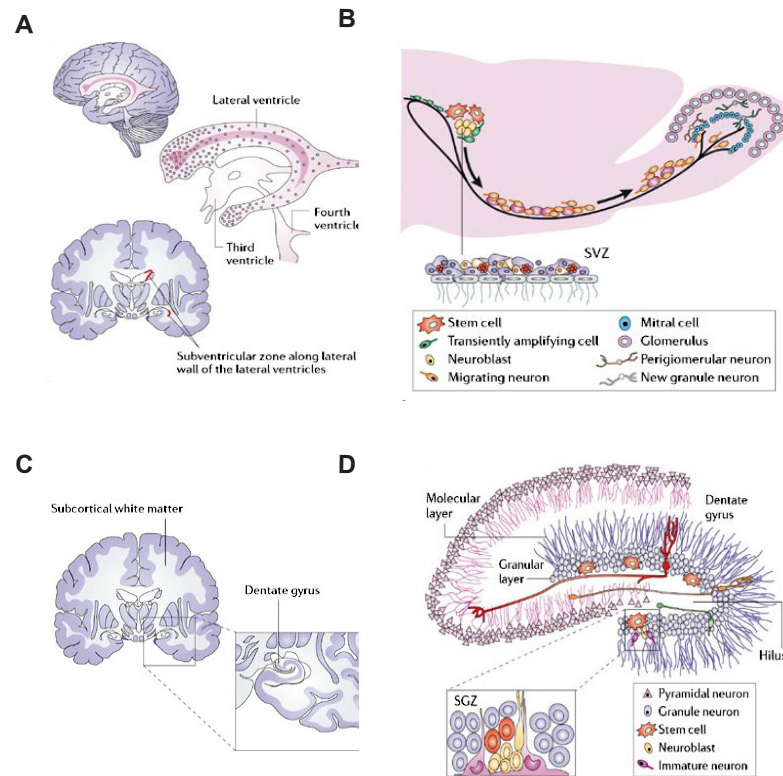


Figure 1.5: NSC reservoirs in the adult human brain.

(A) A sagittal section through the lateral ventricle shows the subventricular zone (SVZ), the larger area of adult neurogenesis. (B) The SVZ is comprised of three main cell types: the multipotent type B astrocytes, which is the neural stem cells that gives rise to fast-cycling transiently proliferating precursor cells that are called type C precursors, which in turn, generate mitotically active type A neuroblasts. The type A cells migrate tangentially towards the olfactory bulbs where they integrate as new interneurons. (C) The subgranular zone (SGZ) is located within the dentate gyrus of the hippocampus. (D) A cellular hierarchy, somewhat similar to that of the SVZ, is seen in the SGZ. The type B astrocyte is the neural stem cell that gives rise to the intermediate type D precursor that eventually gives rise to the type G granule neurons. These neurons integrate functionally into the granule cell layer. Figure reproduced from Vescovi et al., 2006.

ies show that GBM can also arise from terminally differentiated cells through a process of dedifferentiation (Friedmann-Morvinski et al., 2012). NSC-derived progeny such as astrocytes or oligodendrocyte precursors cells (OPC) have also been described as the cell of origin of these tumors (Bachoo et al., 2002; Lindberg et al., 2009; Persson et al., 2010; Liu et al., 2011). OPC can be manipulated *in vitro* to acquire stem-like properties (Kondo and Raff, 2000, 2004). In a murine model of glioma OPC rather than NSC have been suggested as the cell of origin (Liu et al., 2011). In other study, Bachoo *et al.* identified NSC and astrocytes as equally permissive compartments for gliomagenesis (Bachoo et al., 2002).

Furthermore, clinical data reveals that some GBM arise in or near the SVZ adjacent to the lateral ventricle, while other GBM arise in the superficial sub-cortical white matter (Lim et al., 2007; Bohman et al., 2010). Therefore, these data suggest that there might be different cells of origin for GBM, each with different clinical and pathological presentations. Combining currently available evidence from genetic models and clinical observations, it might be proposed that NSC, OPC or astrocytes could be the cell of origin for GBM. In an additional level of complexity, some authors suggest that distinct cells of origin might give rise to each GBM subtype or, alternative, that distinct genetic mutations might transform the same cell of origin into different pathological manifestations (Zong et al., 2012).

1.5 Tumor microenvironment

Several studies have demonstrated the importance of the microenvironment that surrounds tumor cells, which may contribute to tumor initiation and progression, and to the metastatic capacity of cancer cells (Barcellos-Hoff et al., 2013). Important components of the tumor microenvironment include: (1) extracellular matrix (ECM), (2) non-tumor cells in close proximity to tumor cells, such as astrocytes, macrophages, endothelial cells and pericytes, (3) soluble signals, such as growth and differentiation factors and (4) tissue oxygen concentration.

1.5.1 ECM components

The ECM surrounding tumor cells not only serves as a scaffold in which cells growth and migrate, but also plays an important role in tumor behavior. The

main component of brain ECM is hyaluronic acid (HA), which is constitutively produced in GBM (Toole, 2004). CD44, its cellular receptor, is also overexpressed in some GBM, specially in MES-subtype GBM (Wiranowska et al., 2010). Binding of CD44 to HA promotes the activation of pro-tumorigenic pathways that control tumor growth, proliferation, migration and differentiation (Bourguignon, 2008). Fibronectin, laminin and collagen are other matrix proteins in the ECM in the brain. These proteins bind to tumor cell integrins, thereby affecting the cell's behavior. For example, $\alpha 1$ integrin is negatively correlated with drug-induced resistance in GBM (Aoudjit and Vuori, 2001). Additionally, $\alpha 6$ integrin is required for self-renewal and proliferation of GBM stem-like cells (Lathia et al., 2010, 2012). Therefore, adhesion proteins at the surface of the tumor cell that mediate binding to the ECM are crucial players in regulating tumor cell behavior.

At the same time, tumor cells can also remodel the ECM through cell-secreted proteases that enhance tumor progression by removing barriers for cell dissemination. For example, urokinase plasminogen activator (uPA), cathepsin B and matrix metalloproteinases (MMP) are up-regulated in GBM and associated with poor patient prognosis (Kessenbrock et al., 2010; Rao, 2003).

1.5.2 Cell interactions and soluble signals

1.5.2.1 Perivascular niche

NSC are localized to specialized microenvironments within the brain that support their maintenance (Doetsch et al., 2002). The perivascular niche, where NSC reside in close apposition to endothelial cells, has been described as one of the most important NSC niches (Palmer et al., 2000; Shen et al., 2004). The perivascular space is composed of endothelial cells (EC), pericytes and astrocytes (Charles et al., 2011). This niche not only provides an exchange of nutrients and waste products, but also provides a pro-survival environment for these stem cells. Factors secreted by cells in the niche promote NSC self-renewal and keep these cells in an undifferentiated state (Ramírez-Castillejo et al., 2006).

Much like their counterparts, GIC are also present in a perivascular niche which promotes their stem-like properties (Figure 1.6) (Anido et al., 2010; Calabrese et al., 2007; Charles et al., 2010). Nitric oxide (NO) synthesized by endothelial cells stimulates Notch signaling in GIC promoting stem-like character in these

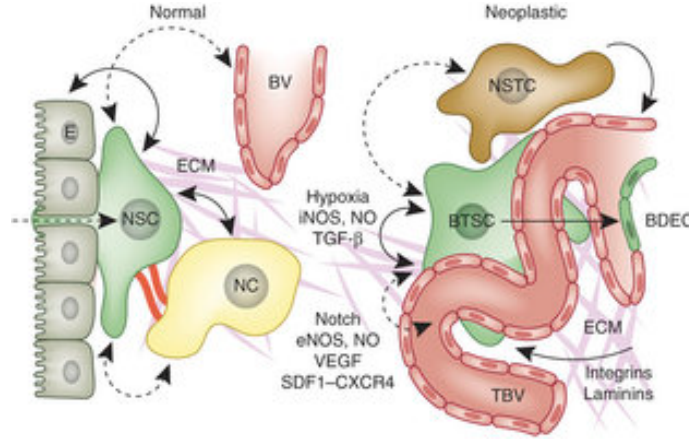


Figure 1.6: *The perivascular space is the niche for NSC and GIC.*

NSC reside in the perivascular niche where they rely on a series of signals between the extracellular matrix (ECM), blood vessels (BV) and other niche cells (NC) to promote their maintenance. Brain tumor stem cells (BTSC) or GIC rely on similar interactions in the perivascular niche which also consists of ECM, non-stem tumor cells (NSTC) and tumor BV (TBV). Figure adapted from Hjelmeland et al., 2011.

cells (Charles et al., 2010). TGF- β secreted by endothelial cells promotes stem-like properties such as self-renewal and tumorigenic potential (Rodón et al., 2014). Astrocytes also deploy autocrine signaling promoting tumor aggressiveness through the secretion of stromal cell-derived factor 1 (SDF-1) and astrocyte elevated gene-1 (AEG-1) (Hoelzinger et al., 2007; Barbero et al., 2002). Other chemokines such as chemoattractant protein-1 (CCL2), interleukin-8 (IL8) and RANTES serve as malignancy-promoting signals in GIC (Zhou et al., 2002). Additionally, GIC also promote proliferation, motility and secretion of MMP from astrocytes (Couldwell et al., 1992; Le et al., 2003).

1.5.2.2 Microglia

At the same time, cancer stem-like cells secrete factors that promote the communication of these cells with their microenvironment. Recent evidences show that recruitment of different immune cells to the tumor niche can modulate cancer stem-like properties (Filatova et al., 2013). Specific factors secreted by the immune cells also alter transcriptomic and epigenetic signatures in the cancer stem-like cells. Microglia release MMP in response to soluble factors secreted

by GIC (Markovic et al., 2005). Tumor necrosis factor alpha (TNF- α) derived from macrophages and/or microglia promotes a phenotypic shift from PN to MES states in GBM (Bhat et al., 2013). Therefore, the crosstalk between tumor cells and their microenvironment enhances and promotes tumor progression. Since there have been several unsuccessful attempts to target tumor cell-intrinsic mechanisms, some authors propose that targeting the microenvironment might be an effective strategy to target the GIC population (Barcellos-Hoff et al., 2013).

1.5.2.3 Hypoxic niche

Hypoxic regions are frequently found in GBM and associated with poor clinical outcome (Rong et al., 2006). Hypoxia has been classically linked to GBM progression and thought to promote a more malignant phenotype of cancer cells (López-Lázaro, 2006). However, hypoxic regions are also the normal microenvironment for NSC (Panchision, 2009). Physiological oxygen concentrations in healthy brain tissue range between 12.5 and 2.5% (physiological microenvironment) whereas GBM hypoxic regions range between 2.5 and 0.5% (hypoxic microenvironment) (Evans et al., 2004). For example, in the hippocampus, where a specific population of adult NSC resides, oxygen partial pressure is 3-4%. Already in 1979, some authors demonstrated that low oxygen tension was required for proper morphogenesis of the cranial neural folds in cultured rat embryos (Morriss and New, 1979). Oxygen levels play an important role in regulating cell fate, proliferation, survival and differentiation in different cell types, including NSC (Ezashi et al., 2005; Morrison et al., 2000; Studer et al., 2000; Zhang et al., 2006). Therefore, oxygen sensing seems to be integrated into signaling pathways controlling normal NSC biology in the brain.

Like their counterparts, GIC also reside in an hypoxic microenvironment which affects tumor function (Pistollato et al., 2010; Seidel et al., 2010). Low oxygen pressure retains the undifferentiated cancer stem-like phenotype enhancing their stem-like properties such as self-renewal (Heddleston et al., 2009; Soeda et al., 2009; McCord et al., 2009). Additionally, hypoxia increases the stem-like Side Population and the percentage of CD133⁺ cells of glioma cells (Bar et al., 2010). However, most functional NSC/GIC studies are performed under normoxic (20% O₂) experimental conditions. Therefore, there might be factors involved in NSC/GIC biology that operate under physiological hypoxic

conditions that might have been underestimated in previous studies due to the experimental conditions used. The study of both NSC and GIC behavior under physiological hypoxic conditions might be required for proper comprehension of NSC and GIC biology.

Hypoxia signaling

Hypoxia is known to affect the transcriptional status of the cell by activating important transcription factor families such as Hypoxia Inducible Factor (HIF) or Nuclear Factor- κ B (NF- κ B), among others. These pivotal factors control a wide range of vital cell functions such as cell fate, proliferation, survival and differentiation (Oliver et al., 2009; Rius et al., 2008; Wang and Semenza, 1993a,b).

The cellular response to hypoxia is mainly controlled by hypoxia-inducible factors 1 α , 2 α and 3 α (HIF-1-3 α) which heterodimerize with a constitutive HIF-1 β assembling a functional heterodimer. The dimerization of these subunits promotes their translocation to the nucleus and transcriptional activation mediated by binding to hypoxia response elements (HRE) (Jiang et al., 1996). HIF-1 β is constitutively expressed in all cell types and is not regulated by oxygen concentration. Among the three inducible subunits, HIF-1 α is the most ubiquitously expressed in many cell types. Under atmospheric oxygen conditions, HIF-1 α is hydroxylated on specific proline residues by prolyl hydroxylase domain protein (PDH). Following hydroxylation, Von Hippel-Lindau (VHL) binds HIF-1 α and targets it for ubiquitination by an ubiquitin ligase complex containing Elongin 3 (E3). After ubiquitination, HIF-1 α is immediately degraded by the 26S proteasome (Figure 1.7) (Kallio et al., 1999; Huang et al., 1998). Under hypoxia, PDH are inhibited which induces the rapid accumulation of HIF-1 α protein, which translocates to the nucleus where it activates transcription of a large set of gene targets.

Under pathological hypoxia, HIF proteins are known to control the transcription of many genes crucial in cancer biology such as angiogenesis, cell survival, glucose metabolism, invasion and metastasis, chemo- and radiotherapy resistance (Semenza, 2010). However, little is known about the role of physiological oxygen concentration on the biology of normal cells. In the case of NSC, only a candidate approach-driven study has identified a couple of HIF targets that are, at least in part, involved in mediating the NSC response to physiological

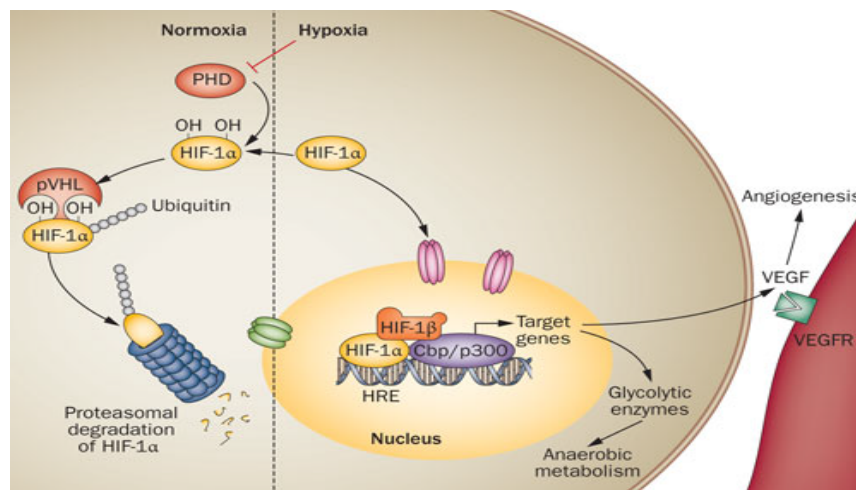


Figure 1.7: *HIF signaling pathway.*

In normoxia, the cellular oxygen sensors prolyl hydroxylase domain-containing protein (PHD) hydroxylate specific proline residues of HIF-1 α , leading to its ubiquitination by the E3 ubiquitin ligase Von Hippel Lindau (VHL) and subsequent proteasomal degradation. During hypoxia, PHD are not active, thereby precluding HIF-1 α ubiquitination and degradation. Stabilized HIF-1 α protein acts as a TF that binds to hypoxia response elements (HRE) to induce the expression of a plethora of target genes, including VEGF. Figure reproduced from Maes et al., 2012.

oxygen concentration. Fibroblast growth factor 8b (FGF8b) partially recapitulates the hypoxia-induced increase in NSC proliferation (Morrison et al., 2000), whereas erythropoietin (EPO) enhances cell proliferation and survival of normoxic human fetal neural progenitors (Morrison et al., 2000; Pavlica et al., 2012). Another study reported that low oxygen levels repress BMP-dependent SMAD activation, thereby inhibiting gliogenic differentiation of NSC (Pistollato et al., 2007).

Despite these few reports, the molecular mechanisms by which physiologic hypoxia controls NSC behavior remain poorly understood. Therefore, there is an urgent need to understand the global impact of sustained physiologic hypoxia on the transcriptional status of NSC. Since GIC share characteristics with their normal counterparts, the NSC, understanding NSC biology in the context of a physiological “hypoxic” niche might help to identify new important regulators also active in stem-like glioma cells.

1.6 GBM tumor progression

Tumor formation involves the co-evolution of tumor cells together with the microenvironment. The complexity of the tumor is a result of a continuous crosstalk between tumor cells and microenvironment over time. Therefore, the dynamic tumor topography varies drastically even through the same lesion. Indeed, most therapies fail as a consequence of the acquisition of a resistant phenotype. Tumor niches shape the responses to the selective pressure of therapy and may affect the emergence of resistance. Therapeutic resistance reflects active tumor evolution and environmental resistance shows the dynamic interplay between tumor cells and their surroundings when the selective pressure of drug is applied. Thus, tumor cell plasticity in response to microenvironment changes has become the main hallmark for success in tumor progression. Consequently, elucidation of how tumor cells adapt to microenvironment changes is essential for the understanding of natural and therapy-driven tumor progression. The gene expression profile of GBM subtypes resembles that of different cell types or tissues, such as brain tissue and cells within the neural lineage including NSC in the PN subtype, or bone, smooth muscle and endothelial cells in the MES subtype (Verhaak et al., 2010; Phillips et al., 2006). Several studies show that elevated cell plasticity is inherent in tumor cells, suggesting that cellular programs that promote cell conversion are playing a major role in tumorigenesis.

1.6.1 EMT in cancer

Cellular plasticity refers to the ability of cells to reversibly change their phenotype. Some cancer cells can reversibly transition between epithelial and mesenchymal states, and some evidences show that cancer cells in the mesenchymal state are more competent at tumor formation than those in the epithelial state (Balic et al., 2006). The transitions between these cell stages are called epithelial to mesenchymal transition (EMT) and, its reverse, mesenchymal to epithelial transition (MET) (Thiery et al., 2009). Both transitions are a common process in embryonic development, wound healing, tissue remodeling, cancer progression and metastasis (Guarino et al., 2007). During EMT, tumor cells lose junctional contacts and epithelial cell polarity while acquiring typical properties of mesenchymal cells. Mesenchymal traits include deposition of ECM components, increased motility and invasiveness and elevated resistance to apoptosis and anoikis (Barrallo-Gimeno and Nieto, 2005; Lo, 2010). However, cancer cells are found in an intermediate stage referred to as an epithelial-mesenchymal hybrid cell (E/M), suggesting that different intermediate stages co-exist within the tumor rather than a unique cell stage (Christiansen and Rajasekaran, 2006).

EMT processes have been extensively described in many different cancer types. In GBM, several EMT inducers have been described as key regulators on the acquisition of malignant features, suggesting that an EMT-like process is also active in GIC (Bhat et al., 2013; Siebzehntrubl et al., 2013; Mikheeva et al., 2010; Myung et al., 2014). The transcriptional network of MES-subtype GBM has been related to classical EMT programs (Carro et al., 2010). Additionally, the PN to MES transition appears to be a common feature during GBM progression. Accordingly, PN-GBM often relapse as tumors with mesenchymal characteristics after radiation therapy in human patients (Phillips et al., 2006). Additionally, a PN to MES transition has also been proposed as a mechanism of acquired resistance to anti-angiogenic therapy (Piao et al., 2004).

1.6.1.1 EMT regulation

The EMT process is controlled at multiple levels including transcriptional regulation, post-translational modifications, cell signaling and epigenetic regulation. One of the crucial steps during EMT is the repression of the major epithelial adhesion molecule E-cadherin, a transmembraneous calcium-dependent

glycoprotein that plays a key role in embryonic development and homeostasis (Desgrosellier and Cheresch, 2010). Downregulation of E-cadherin results in the destabilization of the epithelial architecture and expression of mesenchymal cytoskeletal proteins such as vimentin, and ECM proteins, such as collagen or fibronectin, thereby stimulating the activation of integrins and, subsequently, promoting the migratory capacity of cells.

E-cadherin repressors are classified into different groups based on their direct or indirect repressive action. Direct repressors include *SNAI-1/2*, *ZEB-1/2*, *E47* and *KLF8*, while indirect repressors include *TWIST*, *E2-2* and *FOXC2* (Thiery et al., 2009). Additionally, these repressors also act as direct inducers of EMT by activation of mesenchymal genes such as *CDH2* (N-cadherin), *VIM* (vimentin), *FN1* (fibronectin) and *CCND1* (cyclin D1) (Yang et al., 2004; Perez-Moreno et al., 2001; Zhao et al., 2003). At the same time, these repressors can also inhibit other epithelial genes (Figure 1.8) (Vandewalle et al., 2005; Ikenouchi et al., 2003). Several of these EMT inducers such as *SNAI-1/2*, *ZEB1*, *TWIST1* and *NF- κ B* are active in GBM (Chen et al., 2010; Barbero et al., 2002; Hoelzinger et al., 2007; Couldwell et al., 1992). However, the lack of E-cadherin expression in normal brain and GBM suggests that other mediators might be playing a role in this process (Lewis-Tuffin et al., 2010).

1.6.1.2 EMT-promoting signals

EMT can be initiated by different extracellular signals from the tumor microenvironment. Infiltrated immune cells secrete cytokines, chemokines and growth factors such as *TGF- β* , *TNF- α* , *IL6*, fibroblast growth factor (*FGF*), epidermal growth factor (*EGF*) and hepatocyte growth factor (*HGF*) (Joyce and Pollard, 2009), which are known EMT promoters. The hypoxic tumor microenvironment may also promote EMT through activation of *TWIST* gene expression by *HIF-1 α* (Yang et al., 2008a). Additionally, major embryonic signaling pathways which may be active in tumor cells, such as *WNT*, *SHH* and *NOTCH*, also participate in EMT during cancer progression (Lamouille et al., 2014).

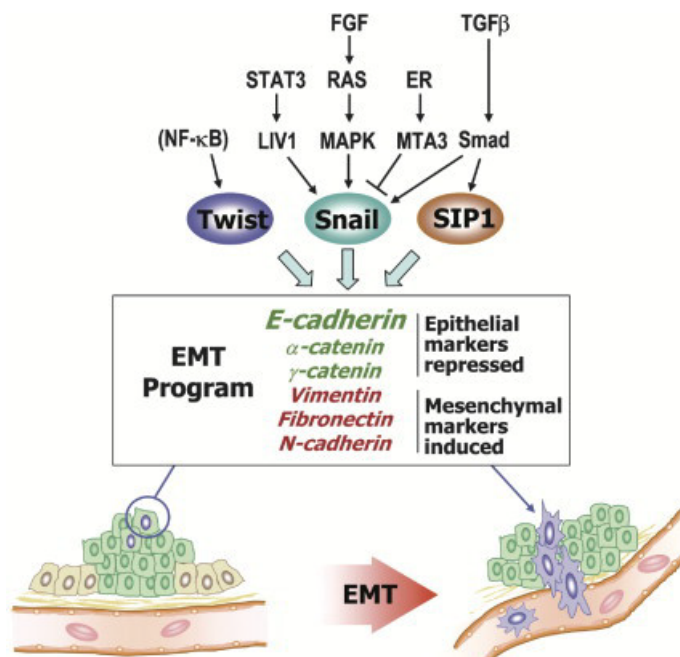


Figure 1.8: *Drivers and mediators of EMT.*

Early stage tumor cells (green) maintain epithelial properties similar to the neighboring normal epithelium (brown). Overexpression of master regulators of EMT, such as *TWIST*, *SNAI* and *SIP1* in cancer cells leads to dramatic changes in their gene expression profile and cellular behavior. These TF repress the expression of E-cadherin via E boxes and trigger the expression of an entire EMT transcriptional program. Several pathways are known to regulate *TWIST*, *SNAI*, and *SIP1* expression in tumor cells. Figure adapted from Kang and Massagué, 2004.

TGF- β

Transforming growth factor- β (TGF- β) is a pleiotropic cytokine that plays critical roles in tumor suppression and tumor progression, cell differentiation and tissue morphogenesis and extracellular matrix production (Massagué, 2008). TGF- β has also been reported as a strong inducer of EMT. TGF- β activates SMAD proteins, which regulate the expression of *SNAI*, *ZEB* and *TWIST* (Xu et al., 2009). Additionally, TGF- β also activates PI3K-AKT cascade and WNT signaling, which significantly contribute to the progression of the EMT process (Jin et al., 2011). In GBM, TGF- β has been shown to promote cell growth, migration, invasion, angiogenesis, immune suppression and stem cell properties (Platten et al., 2001; Joseph et al., 2013; Bruna et al., 2007). More recently, it has been demonstrated that TGF- β can also promote EMT, thereby enhancing GBM tumor invasion (Joseph et al., 2014).

IL-6

Interleukin-6 (IL-6) is a tumor microenvironment-derived factor capable of inducing EMT. IL-6 represses E-cadherin expression and induces the expression of Vimentin, N-cadherin, *TWIST1* and *SNAIL* via the activation of STAT3 (Sullivan et al., 2009). Cancer cell-derived IL-6 is also involved in the activation of cancer-associated fibroblasts tumor (CAF), which stimulates the EMT in cancer cells. In GBM, IL-6 contributes to cancer growth and survival (Giannoni et al., 2010; McFarland et al., 2013; Wang et al., 2009a). IL-6 ligand and receptor are overexpressed in GBM and associated with poor prognosis (Wang et al., 2009a).

TNF- α

Other cytokines such as the tumor necrosis factor α (TNF- α) have also been described as EMT inducers. TNF- α activates tumorigenic pathways leading to cell survival, proliferation, invasion and angiogenesis (Waters et al., 2013). More recently, it has been demonstrated that TNF- α is involved in the EMT process. TNF- α induces EMT by stabilizing SNAIL in colorectal cancer cells (Wang et al., 2013). In GBM, TNF- α induces a shift of proneural GIC into a mesenchymal phenotype by activating the NF- κ B signaling pathway (Bhat et al., 2013).

1.6.1.3 Signaling pathways involved in EMT

NF- κ B signaling

Nuclear factor- κ B (NF- κ B) is a protein complex that controls the transcription of many genes mostly related to immune and inflammatory responses determining developmental process such as cell growth and apoptosis. Five different members compose the mammalian NF- κ B family: p65 (RelA), RelB, c-Rel, NF- κ B1 (p50 and its precursor p105) and NF- κ B2 (p52 and its precursor p100) (Grimm and Baeuerle, 1993). All five members share a Rel homology domain (RHD) which is responsible for DNA binding and dimerization between different or identical family members, building homomeric or heteromeric subunits. NF- κ B TF can be regulated by both canonical and non-canonical pathways.

In the canonical pathway, upstream activators, such as TNF- α , activate the inhibitor of kappa B kinase (IKK) complex which is composed by two catalytic subunits IKK α and IKK β and one regulator subunit IKK γ , also known as NF- κ B essential modulator (NEMO). The activated IKK complex phosphorylates the inhibitor of kappa B (I κ B), which is immediately ubiquitinated and rapidly degraded via the polyubiquitin-dependent proteasomal pathway (Chen et al., 1995). NF- κ B complexes are then released and translocated into the nucleus activating its target genes (Figure 1.9) (Zandi et al., 1998). The alternative pathway is triggered by the I κ B-independent activation of p50 and p52. Upon stimulation, the precursors p105 and p100 are targeted by the preteasome and processed to p50 and p52, which can then translocate to the nucleus and activate transcription of target genes (Figure 1.9) (Sun and Ley, 2008).

Functionally, TNF- α -activated NF- κ B activity correlates with the expression of some EMT inducers, such as *ZEB-1*, *ZEB-2* and *TWIST* (Min et al., 2008). In GBM, the MES subtype has been associated with an hyperactivation of NF- κ B and TNF- α signaling pathways (Verhaak et al., 2010). TNF- α /NF- κ B induces a mesenchymal differentiation of GIC, thereby promoting radioresistance (Bhat et al., 2013).

STAT3 signaling

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT family of latent cytoplasmic transcription factors activated by tyrosine

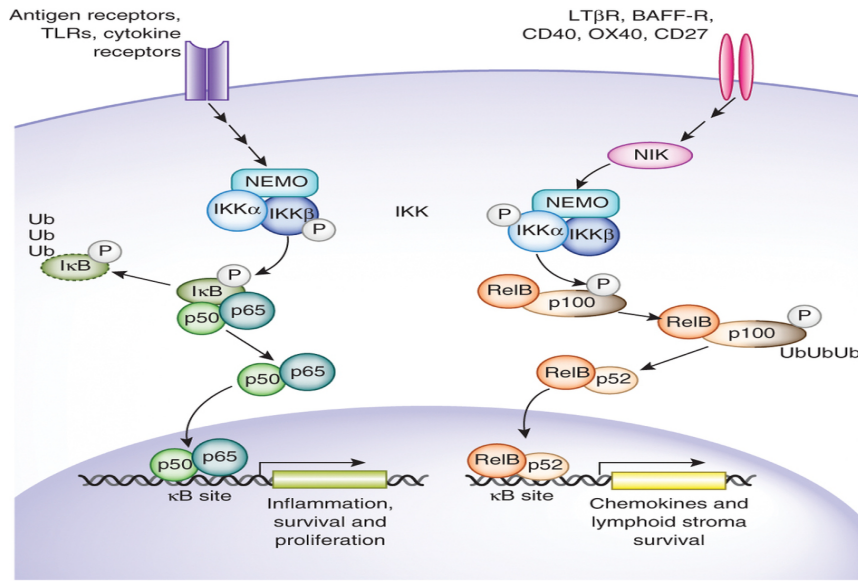


Figure 1.9: *The NF-κB signaling pathway.*

The canonical NF-κB pathway (left) induced by signals including antigens, TLR ligands and cytokines (such as TNF-α) uses a wide variety of signals adaptors to engage and activate the IKKβ subunit of the IKK complex. Activated IKKβ phosphorylates IκBα, which is rapidly degraded by the proteasome. This allows NF-κB to enter to the nucleus where it binds specific DNA sequences that control the transcription of genes involved in inflammation, cell survival and cell division. The noncanonical pathway (right) engaged by members of the TNF-like family of cytokines requires NIK activation to activate IKKα, which phosphorylates p100/p100S, triggering its proteasomal processing required for the activation of transcriptionally active p52/p50-RelB dimers. Figure reproduced from Gerondakis et al., 2014.

kinases in response to inflammatory and proliferative signals (Zong et al., 2012; Bromberg and Darnell, 2000). Such STAT3 activators include cytokines of the IL-6 family (IL-6, IL-11, oncostatin M, LIF, CNTF, IL-27 and IL-35), cytokines of the IL-10 family (IL-10, IL-22, IL-19 and IL-20), EGF family members, HGF, VEGF, IL-23 and IL-21. Upon phosphorylation, STAT3 proteins dimerize and translocate to the nucleus binding to the STAT3-response elements and activating a broad array of target genes (Figure 1.10) (Hirano et al., 2000). STAT3 signaling is also tightly regulated by numerous upstream and downstream checkpoints to ensure an appropriated response to activation.

STAT3 has been described as a driver of several pro-oncogenic mechanisms such as cell proliferation, survival, immune suppression, angiogenesis and invasion. Specifically, STAT3 also promotes EMT in cancer cells. EGF/EGFR signaling induces cancer cell EMT via STAT3-mediated *TWIST* gene expression (Lo et al., 2007). IL-6 can also promote EMT through JAK-STAT3-induced *SNAIL* expression (Yadav et al., 2011). In GBM, a mesenchymal transition can also be induced by STAT3 (Bhat et al., 2011; Carro et al., 2010). Persistent STAT3 activation has been reported in 50%-90% of human cancers (Buettner et al., 2002). Rahaman *et al.* observed constitutive activation of STAT3 in 90% of human GBM tumors (Rahaman et al., 2002). Although *STAT3* gain-of-function mutations have not been reported, numerous alterations in the different levels of STAT3 control have been observed (Buettner et al., 2002).

NF- κ B and STAT3 signaling

Global chromatin binding analysis reveals that STAT3 binds to at least 3,000 different gene promoters and even a larger number of genes also targeted by NF- κ B. Therefore, NF- κ B and STAT3 control both distinct and overlapping groups of genes. Several NF- κ B family members, in particular p65 and p50, can physically interact with STAT3 mutually affecting their transcriptional cell activity (Yu et al., 2002; Yoshida et al., 2004). This non-canonical interaction involving unphosphorylated STAT3 (U-STAT3) and unphosphorylated p65 (U-p65) plays an important role during tumor progression (Figure 1.10) (Yang and Stark, 2008). In GBM, IR promotes the physical association between NF- κ B and STAT3, thereby enhancing migration and invasion (Kesanakurti et al., 2013).

Additionally, other crosstalks between these two pathways have been described. For example, NF- κ B promotes an inflammatory response through secretion of

TNF- α or IL-6, which triggers STAT3 activation (Bhat et al., 2013; Ben-Neriah and Karin, 2011; Hayden and Ghosh, 2012). More recently, Bath *et al.* have shown that TNF- α /NF- κ B activation induces mesenchymal differentiation in GBM via induction of STAT3 (Chen et al., 2013). Therefore, the dialog between these two signaling pathways seems to play a pivotal role in mesenchymal differentiation during tumor progression.

1.6.2 Other types of plasticity

In addition to EMT, cancer cells, including GBM cells, have also been described to undergo other types of conversion from one cell type to another. In normal stem cells, two different mechanisms associated with natural plasticity have been described: dedifferentiation and transdifferentiation. Dedifferentiation involves a cell reverting back to a less-differentiated state from within its own lineage. Otherwise, transdifferentiation takes dedifferentiation a step further and involves cells regressing to a point where they can switch lineages, allowing them to differentiate into a cell type from a different lineage (Jopling et al., 2011). Therefore, both processes imply reversion back to early developmental stages.

Much like their counterparts, cancer stem-like cells can also dedifferentiate and transdifferentiate into other lineages allowing for tumor adaptation and progression. For example, tumors cells in melanoma, prostate cancer, sarcoma or GBM can “transdifferentiate” into cells with endothelial cell (EC) properties, which organize into vessel-like structures and promote tumor growth (Hendrix et al., 2003; Casal et al., 2010; Wang et al., 2010; Scully et al., 2012; Ricci-Vitiani et al., 2010; Soda et al., 2011). In agreement with this data, an embryonic stem cell-like gene expression signature has been associated with poorly differentiated human tumors, including GBM (Ben-Porath et al., 2008). However, how these cancer stem-like cells acquire cell plasticity is still unknown.

1.6.3 GBM cell-of-origin and progression

Finding out the cell of origin of tumors might help to get insights about the acquisition of cell plasticity required for cancer progression. As we have mentioned previously, the cell of origin of GBM is still a matter of discussion. Some

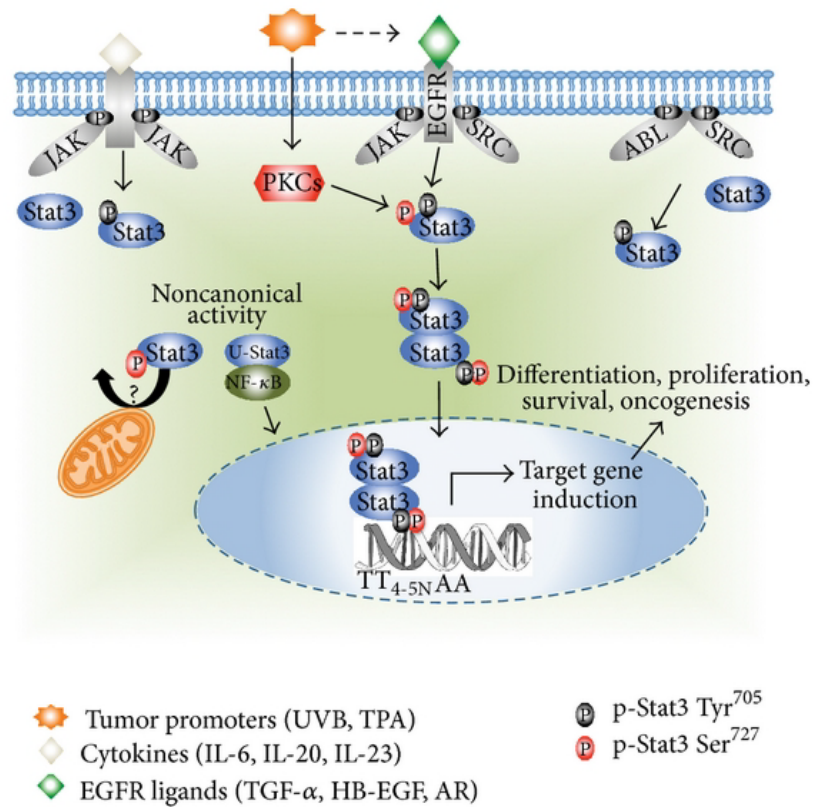


Figure 1.10: *Pathways associated with STAT3 activation.*

(A) STAT3 is activated downstream of receptor tyrosine kinases (such as EGFR), cytokine receptors via associated Janus family kinases (JAK) (such as IL-6 receptor) and nonreceptor-associated tyrosine kinases (such as c-src). After phosphorylation, STAT3 dimerizes and translocates to the nucleus, where STAT3 dimers directly regulate gene expression of transcriptional targets including *BCL-XL*, cyclin D1, *c-MYC*, *TWIST* and Survivin. STAT3-mediated regulation of target gene expression is involved in various cellular functions including cell differentiation, proliferation, survival and oncogenesis. STAT3 can also act through noncanonical signaling pathways. Unphosphorylated STAT3 (U-STAT3) can drive gene expression of a subset of genes that are different from the target genes of p-STAT3 dimers in an NF- κ B-dependent and independent manner. These noncanonical STAT3 signaling pathways have pro-tumorigenic roles in certain cell/tissue types. Figure adapted from Macias et al., 2013.

authors support the idea that NSC are the cells that initiate gliomas (Alcantara Llaguno et al., 2009; Wang et al., 2009b; Zhu et al., 2005). On the other hand, other authors suggest that NSC-derived progeny are also able to initiate glioma tumor growth (Bachoo et al., 2002; Lindberg et al., 2009; Liu et al., 2011; Persson et al., 2010). This unresolved controversy might stem from distinct oncogenic mutations used in the experimental mouse models. However, the inherent plasticity of tumor cells described above might also create confusion about the origin of tumors.

Ozawa *et al.* connected the GBM subtypes in an evolutionary framework where the primordial tumors are PN in phenotype, with the other subgroups evolving from them. They showed that GBM subtypes evolve from a common cancer precursor highlighting the importance of the tumor evolution process (Ozawa et al., 2014). The different GBM subtypes might represent different stages in tumor evolution, with the non-PN subtypes, specially the MES subtype, representing the most advanced stages. These data support the view that dysregulation of specific pathways, rather than cell-of-origin, dictates the phenotype of GBM. Therefore, the key question to be elucidated is which are the cellular programs whose deregulation promotes cellular plasticity, thereby leading to tumor progression.

1.7 Adhesion proteins: GPR56

EMT is a complex cellular process that requires a high level of phenotypic plasticity. During this process either in embryonic development or in tumorigenesis, epithelial cells lose polarity, reduce cellular adhesion and increase cell migration (Thiery et al., 2009; Mani et al., 2008). Loss of cell adhesion is a major hallmark of this process. In a large number of systems, one of the crucial steps in EMT is the repression of the epithelial marker E-cadherin, a transmembraneous calcium-dependent glycoprotein responsible for cell-to-cell adhesion. However, lack of E-cadherin expression in normal brain and GBM suggests that other factors might be playing a role in this process (Lewis-Tuffin et al., 2010). Members of the subfamily of adhesion G-protein-coupled receptors (adhesion GPCR) might be good candidates for such a role.

1.7.1 Adhesion GPCR

G-protein-coupled receptors (GPCR), also known as seven-transmembrane receptors, are integral membrane proteins participating in the transmission of signals from the extracellular environment to the cytoplasm. A variety of external stimuli, such as neurotransmitters, hormones, phospholipids, growth factors and proteases can activate GPCR (Lagerström and Schiöth, 2008). Upon ligand binding, GPCR results in a conformation change that is transmitted to the bound $G\alpha$ subunit of the heterotrimeric GTPase protein. Small GTPases are a family of hydrolase enzymes that cycle between an inactive guanosine diphosphate (GDP)-bound and an active guanosin triphosphate (GTP)-bound state. $G\alpha$ subunit exchanges GTP in place of GDP triggering the dissociation of $G\alpha$ subunit from the $G\beta\gamma$ dimer and allowing them to interact with other proteins to continue the signal transduction (Figure 1.11) (Digby et al., 2006). Small GTPases regulate a wide variety of cellular processes such as cell growth, differentiation and movement. During development, small GTPases play an important role in neural crest EMT and gastrulation (Thiery et al., 2009). Different members of the Rho family of GTPases alter EMT transcriptional programs. For example, RhoV downregulation affects *SNAI2* and *TWIST* expression in *Xenopus* embryo, Raa1 induces *SNAI2* expression in the neural crest of *Xenopus*, RhoA activation abrogates crest cell delamination and RhoB lies downstream of *SNAI2* in the chick neural crest (Guémar et al., 2007; del Barrio and Nieto, 2002; Perez-Alcala et al., 2004; Broders-Bondon et al., 2007). Additionally, several Rho family GTPases members have also been described as essential players for cytoskeletal dynamics during cancer cell migration (Sahai and Marshall, 2002).

The functional relationship between GPCR signaling and EMT has also been extensively described. In cancer, deregulation of GPCR signaling has been implicated in cell migration, invasion and metastasis (Wang et al., 2005; Sahai and Marshall, 2002; Vallon and Essler, 2006; Wu et al., 2013; Tang et al., 2013). For example, the adhesion GPCR GPR116 has been identified as a tumor-promoting receptor that induces breast cancer metastasis (Tang et al., 2013). GPCR constitute more than 40% of the molecular targets of drugs being developed by pharmaceutical industries because they are easily druggable (Tang et al., 2012). However, there are few GPCR that are cancer therapeutic targets. Therefore, studying GPCR function becomes of crucial interest to understand cancer progression and eventually develop novel targeted therapies for the management of

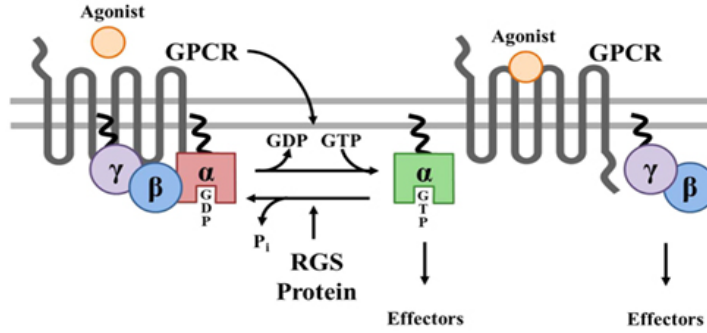


Figure 1.11: *G protein-mediated signaling by GPCR.*

GPCR are seven transmembrane receptors coupled to an intracellular heterotrimeric G-protein (formed by subunits $G\alpha$, $G\beta$ and $G\gamma$). Agonist binding to GPCR induces a conformational change that facilitates the exchange of GDP to GTP on the subunit $G\alpha$. Both GTP-bound $G\alpha$ in the active form and the released $G\beta\gamma$ dimer can go on to stimulate a number of downstream effectors. Figure reproduced from Stewart et al., 2012.

cancer, including GBM.

1.7.2 GPR56

1.7.2.1 GPR56 characteristics

GPR56 is a GPCR that belongs to the subfamily of adhesion GPCR. The human *GPR56* gene is localized on chromosome 16q12.2-q21. The *GPR56* mRNA is alternatively spliced producing multiple mRNA variants that result in three main protein isoforms with the same function (Liu et al., 1999). GPR56 protein contains an extended N-terminus and a seven-pass transmembrane domain. The N-terminus is involved in cell-to-cell and cell-to-ECM adhesion and contains seven N-glycosylation and many O-glycosylation sites indispensable for the cell surface localization of the receptor (Jin et al., 2007). In the intermediate segment, GPR56 is highly enriched in Serine, Threonine and Proline (STP region), which is thought to act as a docking site for O-glycosylation mediating protein-protein interactions (Stacey et al., 2000). GPR56 also contains a cysteine-rich GPCR proteolytic site (GPS) where the receptor is cleaved into two different fragments (Figure 1.12). The GPS motif is required for protein trafficking to

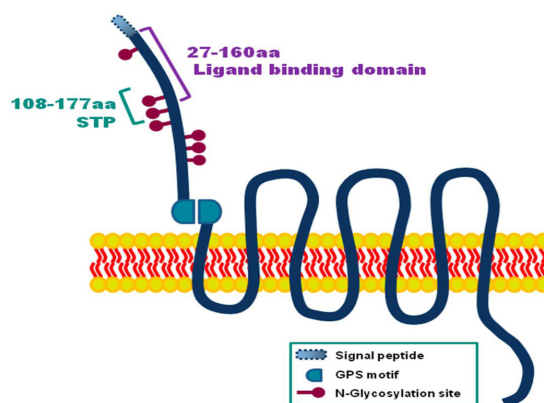


Figure 1.12: *Structure of GPR56 protein.*

GPR56 consists of an exceptionally long extracellular N-terminal region, a GPCR proteolysis site (GPS) and a seven transmembrane (7TM) C-terminal region. The ligand binding domain spans the 27–160 aa, which is essential for binding to collagen III. The Serine-Threonine-Proline (STP)-rich region is the binding site for transglutaminase 2 (TG2). There are a total of seven N-glycosylation sites which are involved in the cell surface localization of the receptor. Figure reproduced from Singer et al., 2013.

the cell surface, being essential for the normal function of GPR56 (Jin et al., 2007). In many GPCR, the C-terminal tail and the third intracellular loop bind to $G\alpha$ proteins and transmit downstream signaling (Figure 1.11). However, this segment is unusually short in GPR56, thereby suggesting that GPR56 might signal through other mediators. Therefore, the molecular mechanism of GPR56 activation is still not clarified. Some authors postulated that the binding of GPR56 with its ligand releases the N-terminus (GPR56N) from the membrane-bound C-terminus (GPR56C) triggering the association of GPR56C with lipids raft, thus activating its downstream signaling molecular RhoA (Luo et al., 2014). Nevertheless, the direct interaction between GPR56 and the $G\alpha$ subunit has not been demonstrated. Accordingly, some authors have hypothesized that GPR56 might activate another GPCR that signals through $G\alpha_{12/13}$ and RhoA proteins (Xu, 2010). Further studies facing the molecular mechanism of GPR56 activation are required for a proper comprehension of the GPR56 function.

1.7.2.2 GPR56 in development

GPR56 is widely expressed in several tissues and organs with the highest expression in brain, thyroid gland and peripheral tissues (Liu et al., 1999). Neural and hematopoietic progenitors express high levels of GPR56 (Piao et al., 2004; Terskikh et al., 2001, 2003). In the brain, GPR56 function is indispensable for normal cortical development. Mutations in the human *GPR56* gene cause a malformed cerebral cortex called bilateral frontoparietal polymicrogyria (BFPP) (Li et al., 2008). This malformation is characterized by an overmigration of neurons beyond a breached pial basement membrane with abnormally numerous and small gyri in the cerebral cortex which is clinically characterized by motor development delay, nonprogressive ataxia, seizures and mental retardation (Piao et al., 2002; Harbord et al., 1990). Knockout mice studies show that GPR56 mediates the attachment of radial glial endfeet to the pial basement membrane (Koirala et al., 2009). Cerebral progenitors that lack GPR56 have an impaired adhesion to laminin, collagen III and fibronectin (Koirala et al., 2009; Luo et al., 2011). As mentioned before, in the developing brain, GPR56 binds to collagen III promoting N-terminus cleavage and release, triggering the association of the C-terminus with membrane lipids rafts by coupling to G α 12/13 and activating RhoA pathway (Luo et al., 2011, 2014).

Recent studies show that GPR56 is also essential for normal neurogenesis since it controls progenitor proliferation in the neocortex (Bae et al., 2014). In the hematopoietic system, GPR56 mediates osteosteal niche interactions in the bone marrow (BM) and maintains hematopoietic stem cells (HSC) in a quiescent state (Saito et al., 2013). Therefore, these data suggest that GPR56 has an important role in stem cell maintenance in both NSC and HSC.

1.7.2.3 GPR56 in cancer

GPR56 has been described up-regulated in some types of cancers compared with their normal counterparts such as breast, ovarian and pancreatic cancers, suggesting that GPR56 might function as an oncogene (Shashidhar et al., 2005; Sud et al., 2006; Ke et al., 2007). However, in other studies, GPR56 seems to play a tumor suppressive role in cancer progression. For example, in gastric cancer, the novel tumor suppressor VEZT up-regulates *GPR56* inhibiting cancer invasion and migration (Miao et al., 2013). In lung adenocarcinoma multidrug resistant

cells, *GPR56* is hypermethylated and consequently downregulated (Guo et al., 2013). Finally, in melanoma, GPR56 act as a metastatic suppressor (Zendman et al., 1999; Xu et al., 2006; Yang et al., 2014, 2011). An antagonistic relationship between GPR56 and transglutaminase 2 (TGM2) inhibits melanoma growth (Yang et al., 2014).

Furthermore, GPR56 was reported to associate with CD81 and Gαq in a complex (Little et al., 2004). Tetraspanin proteins are transmembranous proteins that with the association with other proteins play a pivotal role in cell adhesion and cancer (Hemler, 2008). Given these discrepancies about GPR56 function in cancer, some authors suggest that GPR56 might play different roles in different cancer types or even in different tumor stages.

1.8 NFAT family of transcription factors

The NFAT family of TF consists of four cytoplasmic members (NFATc1, NFATc2, NFATc3 and NFATc4) that share the property of calcium dependent nuclear translocation, and a fifth member (NFATc5), which is constitutively nuclear and calcium independent. The activity of calcium-responsive NFAT is regulated by calcineurin, a calcium/calmodulin-dependent serine/threonine phosphatase. A rise in intracellular Ca^{2+} activates calcineurin (Klee et al., 1979) which rapidly dephosphorylates the four cytoplasmic NFAT (NFATc1-4) (Clipstone and Crabtree, 1992; Flanagan et al., 1991). Dephosphorylation of serines in the amino-termini of NFATc proteins by calcineurin exposes nuclear localization sequences leading to their rapid nuclear import where they bind to their target gene promoters (Figure 1.13). NFATc cytoplasmic subunits require other transcription factors for DNA binding, including AP-1, MEF2 or GATA4, which are usually referred to as nuclear partners (NFATn) (Crabtree and Olson, 2002; Hogan et al., 2003). NFAT signaling performs pleiotropic functions in multiple cell types thereby placing this family of TF as a central hub in the control of developmental as well as oncogenic processes.

1.8.1 NFAT during development

Calcium-regulated NFAT play a key role in orchestrating diverse vertebrate developmental programs, including in immune, central nervous, cardiovascular and

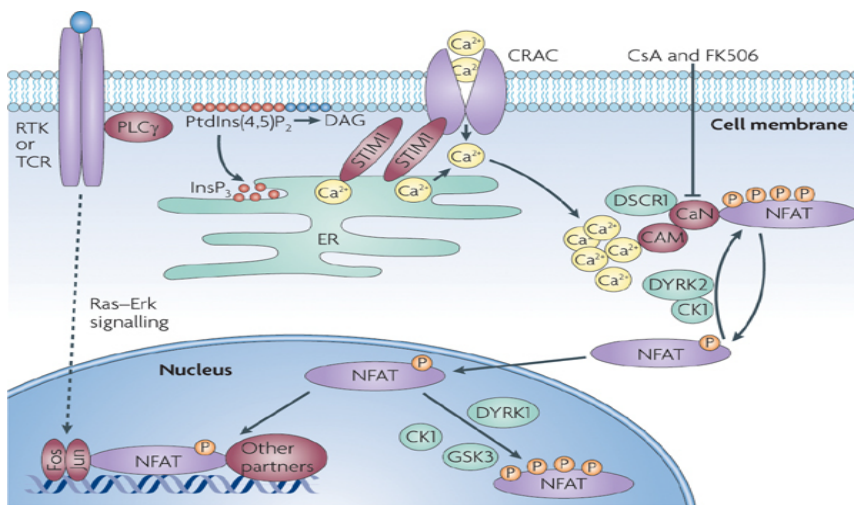


Figure 1.13: *Calcium signaling and NFAT activation.*

Upon calcium entry into the cell, calmodulin (CAM) activates the phosphatase calcineurin (CaN). Inactive cytoplasmic NFAT, which are basally hyperphosphorylated, are rapidly dephosphorylated by calcineurin promoting its nuclear translocation and activation of gene transcription. NFAT cooperate with many other transcription factors, including the activator protein 1 (AP1) and Fos–Jun dimers. Pharmacological antagonists of calcineurin, such as FK506 and cyclosporin A (CsA) are potent inhibitors of NFAT dephosphorylation and nuclear accumulation. The NFAT activation cycle is maintained through complex mechanisms of maintenance kinases that retain hyperphosphorylated NFAT in the cytoplasm, such as casein kinase 1 (CK1) and dual-specificity tyrosine phosphorylation-regulated kinase 1 and 2 (DYRK1/2), as well as nuclear export-promoting kinases such as CK1 and glycogen synthase kinase 3 (GSK3). P, phosphorylation. Figure adapted from Mancini and Toker, 2009.

musculoskeletal systems (Takayanagi et al., 2002; Hogan et al., 2003; Wu et al., 2007; Graef et al., 2003; Winslow et al., 2006; Molkenstein et al., 1998; Ranger et al., 1998; Chang et al., 2004; Chin et al., 1998). These TF control early as well as late developmental processes ranging from early lineage specification in mouse embryonic stem cells (Li et al., 2011) to cardiac valve development (Ranger et al., 1998; de la Pompa et al., 1998). In the nervous system, much is known about the role of NFAT proteins in neurons. Calcineurin-NFAT signaling regulates neuronal survival and maturation, including dendritic/axonal growth and synaptogenesis (Benedito et al., 2005; Graef et al., 1999; Ding et al., 2013; Schwartz et al., 2009). Deletions of three members of the NFAT family, *NFATc2*, *NFATc4* and *NFATc3* cause drastic impairments in neurotrophin/netrin-induced axonal outgrowth (Graef et al., 1999). Additional knockout studies have further shown that calcineurin-NFAT signaling is essential for neuregulin-mediated Schwann cell differentiation (Kao et al., 2009).

Whether NFAT have a functional role in NSC biology is still unknown. Nevertheless, there are some evidences suggesting that calcium signaling might promote NSC properties. Purinergic signaling, which induces intracellular calcium transients, promotes the expansion of embryonic NSC and neurogenesis (Lin et al., 2007). Additionally, two well-known NFATc4-target genes, *FOXP2* and *ID2*, have been described to preserve NSC properties. Forkhead Box Protein P2 (FOXP2) has a crucial role in mammalian neural development by regulating the transition from NSC to transient amplifying precursors (Tsui et al., 2013). Inhibitor of Differentiation/Inhibitor 2 (ID2) preserves the neural stem cell state and prevents neuronal differentiation (Wang et al., 2001; Paoletta et al., 2011; Iavarone and Lasorella, 2006; Ross et al., 2003). However, the direct role of NFATc4 in NSC biology still remains unknown.

1.8.2 NFAT in cancer

The wide range of cellular processes controlled by NFAT proteins and their pivotal role during embryonic development, organogenesis and cell fate determination suggest that this family of transcription factors might also play a role in oncogenesis. Indeed, NFAT isoforms are overexpressed and/or constitutively activated in both solid tumors and hematological malignancies (Mancini and Toker, 2009; Pan et al., 2013). The NFAT family of TF has been shown to regulate cell survival, differentiation, angiogenesis and invasive migration in several

malignancies. Specifically, NFATc2 and NFATc5 have been described as pro-invasive and pro-migratory transcription factors in breast carcinoma, contributing to the formation of metastases (Yoeli-Lerner et al., 2005; Yiu and Toker, 2006). Conversely, NFATc4 downregulation results in actin reorganization associated with increased migration and invasion capabilities in breast cancer cells. NFATc4 signaling reduces migration through the inhibition of Lipocalin 2 (*LCN2*) gene expression (Fougère et al., 2010).

Additionally, NFAT proteins have been identified as microenvironment-related TF since they have been associated with cytokine control (Mancini and Toker, 2009). However, few mediators, such as COX-2, glypican-6, MMP-2, c-Myc, MDM2 and *LCN2*, of the NFAT signaling axis have been identified (Yiu and Toker, 2006; Singh et al., 2010; Fougère et al., 2010). Therefore, there is an urgent need to identify novel upstream regulators and downstream gene targets of NFAT in order to uncover the mechanisms that drive NFAT activation during tumorigenesis.

2

Objectives

The main goal of this PhD thesis was to understand the mechanisms underlying GBM tumor formation and progression, with a special emphasis on the study of factors that have a role in normal NSC biology, which might be providing cancer cells with the stem-like properties required to acquire tumorigenic potential. With this purpose, this PhD thesis was divided into two different projects.

1. Transcriptional profiling of hypoxic NSC identifies calcineurin-NFATc4 signaling as a major regulator of NSC biology.

One of the main goals of our laboratory is to get a deeper understanding of NSC biology as a first step prior to applying this knowledge to the study of gliomas. Therefore, in the first project of this PhD thesis, we set out to identify novel factors that control NSC biology (whose role in NSC was previously unknown). The specific objectives of this project have been:

- * Characterization of the effect of physiologic oxygen concentrations on NSC biology.
- * Identification of novel signaling pathways that are operative in NSC cultured under physiologic oxygen concentrations.
- * Identification of novel TF that orchestrate the NSC response to physiological oxygen concentrations.

- ❖ Functional validation of the role of NFATc4, one of the most promising candidate TF identified above on NSC properties under physiologic oxygen concentrations.
- 2. GPR56 is a NSC factor that restricts the proneural to mesenchymal transition in GBM by inhibiting the NF- κ B pathway.

The second project was focused on the identification of factors that are involved in GBM progression from a PN to a MES subtype (PN-to-MES transition). In a candidate-driven approach, we selected GPR56 as the focus of our studies based on its high enrichment in normal NSC as well as its function as an adhesion receptor. The main objectives of this project are listed below:

- ❖ Characterization of GPR56 expression in the adult mouse brain and during ESC differentiation into the neural lineage.
- ❖ Evaluation of GPR56 expression in the different GBM subtypes and correlation with GBM subtype markers.
- ❖ Functional characterization of the impact of GPR56 knockdown in GIC *in vitro* and *in vivo*.
- ❖ Characterization of the role of GPR56 as an adhesion molecule in GBM.
- ❖ Study of the transcriptional regulation and signaling mediators of the GPR56 pathway.
- ❖ Clinical association studies between GPR56 and the GPR56-associated signature and GBM clinical features, including patient survival and MRI characteristics.
- ❖ Evaluation of the expression of GPR56 and GPR56-associated signature in other tumor types beyond GBM.

3

Materials and Methods

3.1 Plasmids and reagents

Constructs	Specie	Type	Origin
VSVG/His-GPR65	Human	Expression	Dr. Piao
pLKO, shCTRL, shGPR56 and shNEMO	Human	Lentiviral	Mission shRNA Library (Sigma)
STAT3D	Human	Retroviral	Dr. Nakajima (Nakajima et al., 1996)
STAT3C	Human	Lentiviral	Dr. Bromberg (Bromberg et al., 1999)
NF- κ B-Luc reporter	Human	Expression	Dr. Bonni
9XNFAT-Luc reporter	Human	Expression	Dr. Wilkins (Wilkins et al., 2004)
CTRL and NFATc4 siRNA	Human	Expression	Life Technologies
NULL and IKB-SR	Human	Adenoviral	Vector Biolabs
pCDH-GFP	Human	Lentiviral	Dr. Konopka
CAG-GFP	Mouse	Retroviral	Dr. Borrell

3.2 Cell cultures

3.2.1 Cell lines

Glioma initiating cells (GIC) are GBM patient-derived stem-like cells lines obtained from different collaborators (see table below). GIC were cultured in laminin-coated cell plates (10 μ g/ml; Invitrogen) and maintained in Dulbecco's

Materials and Methods

Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Invitrogen) supplemented with N-2 Supplement (Life Technologies), bFGF (20 ng/ml; Life Technologies) and EGF (20 ng/ml; Life Technologies). GIC were treated with TNF- α (10 ng/ml; Prepotech) with different durations of time, as indicated in figure legends. GIC were cultured at 37°C, 5% CO₂ and 5% oxygen.

For endothelial cells (EC), plates were pre-coated with polyornithine (15 ng/ml; Sigma) for 1h at 37°C before seeding the cells. Human brain microvascular endothelial cells (BMEC) were maintained in a special endothelial cell medium (ECM; Innoprot) with 5% FBS and endothelial cell growth supplement (ECGS; Innoprot) following manufacturer's instructions. Human umbilical vein endothelial cells (HUVEC) were kept with endothelial cell growth media (EGM-2; Lonza) supplemented with the endothelial cell growth supplement (EGM2-Bulletkit; Lonza) following the manufacturer's instruction. EC were maintained at 37°C, 5% CO₂ and 20% O₂.

293T-HEK and U87-MG were cultured using Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen). Cells were kept at 37°C in a humidified atmosphere containing 5% CO₂ and 20% O₂.

Before reaching the confluence, cell monolayers were rinsed with phosphate-buffered saline (PBS; Life Technologies) and incubated with Trypsin-like Enzyme (TripLE; Life Technologies) for 10 minutes at 37°C. Complete medium was added and cells were subsequently diluted in fresh medium. Cells stably infected with retroviral or lentiviral plasmids were selected according to its resistance with Puromycin (1 μ g/ml; Sigma Aldrich) or Neomycin (700 μ g/ml; Invitrogen).

Neural stem cells (NSC) were isolated from E13.5 mouse embryos (OF-1 strain) as previously described (Johe et al., 1996). NSC were cultured both in laminin-coated cell plates (10 μ g/ml; Invitrogen) and in suspension as neurospheres. After isolation, cells were immediately cultured at 37°C, 5% CO₂ and either 5% or atmospheric oxygen ($\approx$ 20%) and maintained in these conditions for 2-3 passages. NSC were maintained as described in GIC.

Lateral ganglionic eminences (LGE) from E14.5 mice were dissected out and mechanically disaggregated as previously described (Martín-Ibáñez et al., 2010). Briefly, LGE-derived neurosphere cultures were obtained by seeding 50,000 cells/cm² in complete medium (DMEM:F12; Sigma:Invitrogen, 0.3% glucose

(Sigma), 0.3 mg/ml glutamine (Invitrogen), 5 mM HEPES (Invitrogen), 100 U/ml penicillin, 100 mg/ml streptomycin (Invitrogen), 4 µg/ml heparin (Sigma Chemical), 4 mg/ml Bovine Serum Albumin (BSA) (Sigma Chemical), N-2 Supplement (Invitrogen). Basal medium was supplemented with 20 ng/ml fibroblast growth factor (FGF; Sigma) and 10 ng/ml epidermal growth factor (EGF; Invitrogen).

Cell line	Cell type	Origin	Medium
GIC2	Human glioblastoma	Dr. Alonso (CIMA, Pamplona)	DMEM/F12
GIC4-7	Human glioblastoma	Dr. Gómez-Manzano/ Dr. Juan Fueyo (MD Anderson Cancer Center, Houston)	DMEM/F12
GIC8-11,13	Human glioblastoma	Dr. Galli (Università Vita-Salute San Raffaele, Milan)	DMEM/F12
U87	Human glioblastoma	ATCC	DMEM
293T-HEK	Human kidney	Clontech	DMEM
BMEC	Human endothelial cell	Innoprot	ECM
HUVEC	Human endothelial cell	Lonza	EGM-2

H9 human embryonic stem cells (ESC) were obtained from the WiCell Research Institute (Madison, Wisconsin, USA). For maintenance, they were cultivated on Matrigel (Stem Cell Technologies) coated 6 well plates in mTeSR1 medium (Stem Cell Technologies). The cells were passaged when they reached 70-80% confluence. Cells were washed once with PBS without calcium and magnesium then, 1 ml of 1 mg/ml dispase (Stem Cell Technologies) supplemented with 10 µM ROCK inhibitor (Y-27632) and incubated at 37°C/5% CO₂ for 20-25 minutes. The dispase was aspirated and 6 ml of DMEM:F12 (Invitrogen) was used to harvest the cells. Then, cells were centrifuged at 1000 rpm for 3 minutes, the supernatant was aspirated and the cell pellet was resuspended in 200 µL mTeSR1 + 10 µM ROCK inhibitor. Cell suspension was transferred to a Matrigel coated well of a 6 well plate already containing fresh warm mTeSR1 + 10 µM ROCK inhibitor. 24 hours after passaging medium was changed to fresh mTeSR1 without ROCK inhibitor and then was changed every 24 hours

after that until the cells required passaging again. H9 ESC were differentiated until 28 days *in vitro* (DIV) following the telencephalic differentiation protocol described in (Telezhkin V. et al., in preparation).

Human mesenchymal stem cells (hMSC) were derived from adipose tissue as previously described (García-Olmo et al., 2005; Vicente López et al., 2011). Briefly, cells were obtained by elective liposuction procedures and isolated in DMEM high glucose (Invitrogen) with 10% FBS, penicillin and streptomycin. hMSC were maintained and expanded in a 5% CO₂-in-air incubator at 37°C and experiments were performed with cells harvested at third passage. Mouse mesenchymal stem cells (mMSC) were derived from the bone marrow of femur and tibia from C57/BL6 mice. Cells were cultured in DMEM high glucose (Invitrogen) with 15% FBS, penicillin and streptomycin and harvested at third passage.

3.2.2 Cell transduction

3.2.2.1 DNA transfection

For luciferase assays, transfection using the GenJet Plus Reagent (SignaGen Laboratories) was performed in 24-well plates using 1500 ng of pLuc vector co-transfected together with 26 ng of Renilla luciferase vector. DNA expression vector was transfected at 1500 ng per well. GenJet Plus Reagent was diluted with DMEM/F12. Liposomal mixture was left for 15 minutes at room temperature and afterwards, the solution was added to plated cells at 90% confluence. After 4 hours, medium was replaced with fresh medium. Luciferase assays were conducted at 48 hours post-transfection as stated in results.

3.2.2.2 DNA electroporation

Transient electroporation was performed in 6-well plates using 3000 ng of the different DNA vectors using the Nucleofector II device (Lonza). In the *NFATc4* silencing experiments, NSC were dissociated at a single-cell level and electroporated with the different siRNAs (90pmol; Life Technologies) with the Nucleofector II device (Lonza).

3.2.2.3 Lentiviral and retroviral infection

Lentiviral vectors were generated by co-transfection of the plasmids of interest plus viral packaging vectors. 293T-HEK were cultured in 100 mm cell plates ($\approx$ 90% confluence). Cells were transfected with the three-lentiviral packaging plasmids (VSVG, RSV-REV and pMDL g/p RRE) using the GeneJet Plus DNA transfection Reagent (SignaGen Laboratories). For recombinant retroviruses, the plasmids of interest were co-transfected with the two-retroviral packaging plasmids (pMD.MLV gag.pol and VSVG), as described above. At 24 and 48 hours post-transfection, culture supernatants were collected and purified by centrifugation at 40,000 g for 2 hours at 4°C. Pellets were resuspended in PBS and stored at -80°C until use.

For infection, cells were treated with 100 μ l of viral suspension for 4h. Cells were then washed and fresh medium was added. 48h later, infected cells were selected with the appropriate drug selection.

3.2.2.4 Adenoviral infection

Null or IxB-SR adenoviral particles were purchased from Vector Biolabs. Cells were infected with 10 PFU/cell. Cells were then washed and fresh medium was added.

3.2.3 Flow cytometry

3.2.3.1 Flow cytometry

Approximately 10^6 cells per each condition were collected, rinsed with PBS and blocked with PBS-BSA 1% for 20 minutes at 4°C. Then, cells were stained with a FITC-conjugated anti-CD44 (1/50; BD Pharmingen) for 1 hour at 4°C. After wash, cells were resuspended with PBS-BSA 1% at a final concentration of approximately of 10^6 cells/ml. Cells were immediately assessed by flow cytometry using FACSCanto (Becton Dickinson). Analysis was performed using FlowJo software (Miltenyi Biotec).

For co-culture experiments, 10^5 cells of each cell type were seeded for 8 days. Cells were stained for anti-MCAM (1/20; Merck Millipore) for 1 hour at 4°C, as previously described. APC secondary antibody was used and incubated for

1 hour at 4°C (1/100; Miltenyi Biotec). Cells were assessed using FACSCanto (Becton Dickinson) and analyzed with FlowJo software (Miltenyi Biotec).

3.2.3.2 Fluorescence-activated cell sorting (FACS)

For Fluorescence-Activated Cell Sorting (FACS), cells were stained for CD44 as described below and also treated with LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen). The stained cells were sorted using a FACS Aria cell sorter (Becton Dickinson). Analysis was performed using FlowJo software (Miltenyi Biotec).

3.2.3.3 Image Stream Imaging Flow Cytometry (AMNIS)

Co-culture experiments described above were stained for MCAM for 1 hour at 4°C (1/20; Merck Millipore). After wash, secondary AlexaFluor 594 (Invitrogen) was incubated for 1 hour at 4°C. Cells were resuspended with PBS-BSA 1% at a final concentration of approximately of $2 \cdot 10^7$ cells/ml. Cells were immediately analyzed using Image Stream Imaging Flow Cytometry (Millipore).

3.2.4 Cell cycle analysis

Cells were fixed with 2 ml of 70% pre-cooled ethanol. After overnight fixation, cells were washed and re-suspend with PBS containing 30 µl of RNase A (10 mg/ml; Thermo Scientific) and incubated at 37°C for 30 minutes. Then, cells were treated with 30 µl of propidium iodide (1 mg/ml; Life Technologies) and cell cycle was performed using the FACSCanto cytometer and analyzed using FACSDiva software (BD Bioscience).

3.2.5 Self-renewal assays

NSC maintained in normoxia (atmospheric oxygen) or hypoxia (5% oxygen) were mechanically dissociated at a single-cell level, sorted through a FACS Aria cell sorter (Becton Dickinson) and viable cells (labeled using the LIVE/DEAD Fixable Dead Cell Stain Kit; Life Technologies) plated at low density (50 cells/well of a 96 multi-well plate).

In the calcineurin inhibitor experiments, NSC were dissociated at a single-cell level, seeded at low density and treated with different concentrations of Cyclosporin A (CsA) plus Tacrolimus (FK-506) (4 μ M and 8 μ M) or vehicle control (DMSO). In the *NFATc4* silencing experiments, NSC were dissociated at a single-cell level, electroporated with the different siRNAs using the Nucleofector II device (Lonza), labeled for viability, sorted through a FACSaria cell sorter (Becton Dickinson) and plated at low density. Neurosphere formation was assessed 7 days after plating.

3.2.6 Proliferation assays

1·10⁶ cells were seeded into p60 plates and after 24 hour they were treated with 50 μ M of 5-ethynyl-2'-deoxyuridine (EdU) for 30 minutes. Then, cells were collected and stained following the Click-iT EdU Flow Cytometry Assay Kit instructions (Invitrogen). Briefly, cells were fixed for 15 minutes at room temperature with Click-iT fixative component D and washed with PBS-BSA 1%. Then, cells were permeabilized for 15 minutes with the Click-iT saponin-based permeabilization and wash reagent. Finally, cells were stained with the Click-iT reaction cocktail following the manufacturer's instructions and the stained cells were analyzed using a FACSCalibur cytometer (BD Bioscience).

3.2.7 Immunofluorescence

Cells were seeded in laminin pre-coated coverslides and treated if necessary. Cells were rinsed and fixed with freshly 4% paraformaldehyde (PFA) during 20 minutes at room temperature. Then, cells were permeabilized with PBS-triton X-100 (0.4%) for 15 minutes at room temperature. Unspecific interactions were blocked by incubating cells with 3% BSA in TBS-Tween 0.2% plus 1% Goat Serum during 1 hour. Primary antibody was added and incubated for 1 hour at room temperature. Then, primary antibody was washed with PBS and secondary fluorescent antibody was added (Alexa Fluor 488 and 594; Life Technologies) for 1 hour at room temperature. Cells were washed and coverslides were mounted with Fluoroshield with DAPI (Sigma). Immunofluorescence samples were assessed under fluorescence microscope or stored at 4°C protected from light. Immunofluorescence slides were visualized using Confocal TCS SP5 (Leica). Fiji software was used for fluorescence quantification.

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Antigen	Host	Manufacturer	Dilution
NFATc1	Mouse	Novus Biologicals	1/100
NFATc2	Rabbit	Cell Signaling	1/50
NFATc3	Rabbit	Bioss Antibodies	1/100
NFATc4	Rabbit	Affinity BioReagents (Pierce)	1/100
RelA/p65	Rabbit	Cell Signaling	1/250
GFP-FITC	Goat	Abcam	1/100

3.2.8 γ -H2AX assays

Cells were placed in coverslips coated with laminin and treated with 3 gray (Gy) of ionizing radiation (IR). At the indicated times, cells were fixed with 4% PFA in PBS buffer and immunostained for γ -H2AX to visualize foci formation. Briefly, after blocking unspecific unions with Normal Goat Serum (NGS) 1/10 in PBS-Tween (0,1%) for 2 hours, cells were incubated overnight (O/N) with an antibody anti- γ -H2AX (1/400; Millipore) in 1/100 NGS/PBS-Tween (0,1%), followed by a 1,5 hour incubation with a CyTM3-conjugated F(ab')₂ Donkey anti-mouse IgG as secondary antibody (1/500; Jackson ImmunoResearch) in NGS 1:100/PBS-Tween (0,1%). Nuclei were counterstained with DRAQ5 (1/2500; Biostatus). Images were acquired with a Spectral Confocal Microscope (TCS-SL; Leica Microsystems) using the accompanying image processing software from Cytovision. Nuclei and foci were counted using Image J software (U.S. National Institutes of Health). Between five and eight images were analyzed for each treatment condition.

3.2.9 Cell adhesion assays

3.2.9.1 Cell adhesion assays on matrigel matrix

96 well-plate were coated with 30 μ l of Matrigel (Stem Cell Technologies) and incubated at 37°C for 30 minutes. 8,000 cells were seeded and conditioned medium was added. Cells were monitored for 48 and 72 hours.

3.2.9.2 Cell adhesion assays on endothelial cells

Endothelial cells were seeded in polyornithine pre-coated 6 well-plates. When 100% confluence was reached, $2 \cdot 10^5$ GFP-tagged GIC were plated on top of the

endothelial monolayer for 1 hour at 37°C. Cells were then rinsed with PBS, washed twice and trypsinized. The number of GFP⁺ cells that had adhered to the endothelial monolayer were assessed by flow cytometry using FACSCanto (Becton Dickinson) and analyzed using FACSDiva software (BD Bioscience).

3.3 Protein manipulation

3.3.0.3 Western blot

Cells were lysed using 10% NP-40 buffer supplemented with protease inhibitor cocktail (Sigma) at 4°C for 20 minutes. Protein extract was quantified using Bradford due-binding method (Protein Assay; Bio-Rad Laboratories) by measuring the absorbance at 595 nm with a spectrophotometer (Sinergy HT; BioTek). Cells extracts were separated on a 10% polyacrylamide gel and transferred to PVDF membrane. The membrane was incubated with a specific blocking buffer (Odyssey blocking Buffer; LI-COR Biosciences) for 1 hour at room temperature. Primary antibody was incubated during an O/N at 4°C in constant agitation. The primary antibody was washed 3 times for 5 minutes. Secondary fluorescent antibody was incubated for 1 hour at room temperature, as follows: donkey anti-mouse, goat anti-rabbit, donkey anti-goat, and anti-sheep IRDye 800CW or IRDye 680LT (LI-COR Biosciences). Membrane was washed again and target proteins were detected using the ODYSSEY Infrared Imaging System (LI-COR Biosciences) following the manufacturer's instructions. Analysis was performed using Image Studio Software (LI-COR Biosciences).

Antigen	Host	Manufacturer	Dilution
GPR56	Goat	R&D	1/500
IxB	Rabbit	Santa Cruz Biotechnology	1/100
STAT3	Mouse	BD Transduction Laboratories	1/1000
Phospho-STAT3	Mouse	Cell Signaling	1/2000
p65	Rabbit	Cell Signaling	1/1000
GAPDH	Mouse	Ambion	1/40000
Tubulin	Mouse	Cell Signaling	1/10000
NFATc4	Rabbit	Affinity BioReagent (Pierce)	1/250
NEMO	Rabbit	Santa Cruz Biotechnology	1/1000

3.3.1 Luciferase assays

Luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System following the manufacturer's instructions (Promega). Briefly, cell extracts were obtained from PBS-rinsed cell cultures with passive lysis buffer (PLB) by gentle agitation during 20 minutes and, immediately, lysates were briefly centrifuged to discard cell debris. Firefly luciferase activity was measured adding 25 μ l of luciferin (LARII) to each sample. After 2 seconds of premeasurement delay, firefly luciferase activity was measured for 10 seconds. Then, 25 μ l of coelenterazine (Stop&Glo) was added and renilla luciferase activity was measured. The spectrophotometer Synergy HT was used to measure the luciferase activity (BioTek).

3.4 RNA manipulation

3.4.1 RNA extraction

For RNA extraction, cells were rinsed with PBS and lysed in RLT buffer (RNasy Mini Kit; Qiagen). Total RNA was immediately extracted according the manufacturer's instructions. RNA concentration and quality was assessed with a Nanodrop (Thermo Scientific). RNA quality control for microarray analysis was performed in a 2100 Bioanalyzer (Agilent Technologies).

3.4.2 RT-qPCR

A reverse transcription was performed using 1 μ g of the total RNA by using the High Capacity cDNA Reverse Transcription Kit according to the manufacturer's recommendations (Applied Biosystems).

mRNA Reverse Transcription Conditions

1. 25°C for 10'
2. 37°C for 2h
3. 4°C

The quantitative real time PCR (qPCR) was performed in the StepOnePlus Real-time PCR Systems (Applied Biosystems) using TaqMan Fast Universal

PCR Master Mix (Applied Biosystems) and TaqMan probes (Life Technologies). For human samples the following TaqMan probes were used: TGFB1, FN1, CTGF, ZEB1, TIMP1, CD44, CHI3L1, SERPINE1, SNAIL1, SNAIL2, MCAM, TAZ (to define the MES phenotype); OLIG2, SOX2 and SOX9 (to define the PN phenotype); hGPR56; and GAPDH as a housekeeping gene. Mouse TaqMan probes used were: NFATc1-4, HIF3A, VEGF, NDUF4L2, GABRA3, CAMK1G, TRIM47, ID2, FOXP2, and PMSC4 and IPO8 as housekeeping genes.

mRNA qPCR conditions

1. 95°C for 20'
2. 95°C for 1'
3. 60°C for 20'
4. Go to 2 x 39 cycles

The ΔCT value was obtained by subtracting the CT value of housekeeping genes to the CT value of the targeted genes. The relative mRNA expression level of the target genes was obtained by the formula $2^{-\Delta CT}$.

3.4.3 Microarray analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen). RNA quality control was performed in a 2100 Bioanalyzer (Agilent Technologies). In all samples, the RNA integrity number was > 7 . Complementary RNA were prepared for hybridization in a SurePrint G3 Mouse GE 8x60k array (hypoxic *versus* normoxic NSC study) or G3 Human GE 8x60k array (*GPR56* knockdown-GIC signature), both from Agilent Technologies, using the One-Color Low Input Quick Amp labeling protocol according to the manufacturer's instructions (Agilent Technologies).

3.5 DNA techniques

3.5.1 Transformation into competent cells (DH5 α)

Plasmid DNA amplifications were carried out after their introduction in chemically competent *E. coli* cells. Transformation of competent *E. coli* cells was carried out following the manufacturer's instructions (Subcloning Efficiency DH5

Competent Cells; Invitrogen). Briefly, cells were incubated with the DNA on ice for 30 minutes. Then, cells were placed at 45°C for 90 seconds. 500 µl of XY Medium (2xYT Medium; Sigma) were added and incubated at 37°C for 1 hour. After a briefly centrifugation, transformed cells were spread on pre-warmed selective plates during an overnight at 37°C. For large-scale isolation of plasmid DNA, commercial maxipreparations were used following the manufacturer's instructions (NucleoBond Xtra Maxi; Macherey-Nagel). DNA determinations were made using a spectrophotometer (Nanodrop; Thermo Scientific).

3.6 Animal manipulation

3.6.1 Intracranial injections

1.2·10⁵ GIC stably expressing luciferase were resuspended in 3 µl of PBS and kept on ice. 4-6-weeks old athymic nude mice were anesthetized with intraperitoneal administration of Ketamine/Xylazine (100 mg/kg and 10 mg/kg, respectively). Head skin of each mouse was cut with scissors to expose the skull. Each mouse was situated in the stereotactic and immobilized. A small hole to the skull was carefully performed with an 18G needle at coordinates 0.5 mm lateral / 2 mm anterior from bregma. Cells were inoculated into the brain at 3 mm of depth using a Hamilton 30 G syringe. Incision on head skin was closed with 5 silk sutures and mice were injected subcutaneously analgesic Buprenorphine (0.05 mg/kg).

3.6.2 Luciferase *in vivo*

GIC stably expressing luciferase were injected orthotopically into the brains of nude mice. Intracranial tumor growth was monitored using non-invasive bioluminescence imaging approximately 8 weeks after tumor cell implantation. Briefly, D-luciferin (75 mg/kg; AnaSpec, Inc) was administered intraperitoneally 10 minutes before imaging. Then, mice were anesthetized with isofluorane and imaged using the Aequoria MDS system (Hamamatsu). When appropriate, mice were sacrificed and brain sections were stained with hematoxylin and eosin (H&E) and subject to immunohistochemistry (IHC, details below).

3.6.3 *In utero* electroporation

In utero electroporation was performed as described previously (Borrell, Yoshimura, Callaway 2006). A mixture of DNA constructs was injected in the lateral telencephalic ventricle of E13.5 mouse embryos at the following concentrations: CAG-EGFP, 0.7 $\mu\text{g}/\mu\text{l}$; NFATc4 siRNA, 200 μM ; control siRNA, 200 μM . BrdU was injected intraperitoneal in electroporated embryos 30 min or 24 hours prior to analysis. Embryos were then fixed, cryosectioned and stained. Primary antibodies were anti-BrdU (1:200; Abcam), anti-Ki67 (1:200; Novocastra), anti-GFP (1:1000; Aves Labs), followed by the appropriate fluorescently-conjugated secondary antibodies (1:200; Jackson), and finally counterstained with DAPI (SIGMA). For BrdU and Ki67 detection, sections were pre-treated with 2N hydrochloric acid (HCl) at 37°C during 30 minutes.

3.7 Histological techniques

Adult mouse (P28) brains were perfused with cold PBS at low flow rate until PBS is clear (1-2 minutes), switched to 4% PFA and perfused for 2-3 mins (5-6ml). Then, brains were removed and fixed in 4% PFA O/N at 4°C. After that, cells were switched to 30% sucrose and after brain sinks to the bottom of tube, samples were embeded in OCT, freezed in dry ice and 100% ethanol, wrapped in parafilm and stored at -80°C.

Slides (12 μm thick) were put in retrieval solution (Retrievagen A; BD Biosciences-Retrievagen A pH) at 4°C and immediately they were boiled for 12 minutes. Then, slides were cooled down for 30 minutes at room temperature and washed with PBS for 5 minutes. Slides were treated with 0.5% SDS/PBS for 5 minutes and washed with PBS for 5 minutes. Blocking with 1% BSA and 10% goat serum in PBS containing 0.1% Triton-X 100 was added and incubated at room temperature for 1 hour. Primary antibodies were incubated in 1% BSA and 10% goat serum in PBS containing 0.1% Triton-X 100/PBS at 4°C O/N and then washed with PBS for 5 minutes. Slides were also washed with high salt PBS (2.7% NaCl) twice for 5 minutes each. Secondary antibody was incubated (goat anti-mouse 488; Invitrogen) in PBS containing 0.1% Triton X-100, 1% BSA and 10% goat serum for 1 hour at room temperature. Then, slides were washed with PBS for 5 minutes, dyed with DAPI (1:2000) for 3 minutes at room temperature and mounted.

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For tumor-bearing brains from mouse orthotopically injected with GIC, mice were perfused with 4% PFA. Whole brains were then removed from the skull and post-fixed O/N with 4% PFA. Brains were washed once with PBS and embedded in paraffin. For human GBM, tumor specimens were fixed in formol and included in paraffin for long-term storage.

Both mouse and human paraffin-embedded tumor specimens were cut at 3 microns in a microtome and tumor slides were placed in DAKO⁺ charged slides. Slides were heated at 65°C for 3 hours to O/N and deparafinized in a series of 3 xylenes. Then, slides were hydrated in a series of decreasing ethanols (100%, 90% and 70%) and distilled water. Heat-mediated antigen retrieval was performed using boiling citrate buffer for 20 minutes.

Peroxidase was blocked with 3% hydrogen peroxide (H₂O₂) solution for 10 minutes protected from light. Slides were rinsed with TBS-Tween 10% (3 washes for 5 minutes). Samples were incubated with blocking solution (2% BSA and 10% goat serum) for 30 minutes at room temperature in a humid chamber. Then, slides were rinsed again with TBS-Tween and incubated with the primary antibody diluted in the appropriate buffer at 4°C during O/N in a humid chamber.

Next day, primary antibody was rinsed in 3 washes with TBS-Tween and secondary HRP-conjugated antibody (EnVision; Dako) was added and incubated for 20 minutes at room temperature in a humid chamber. Slides were rinsed in 3 washes and developed with diaminobenzidine (DAB). Samples were then dehydrated in a series of ethanols (70%, 90%, 100%) and 3 clean xylene solutions. Samples were mounted using DPX mounting medium.

Antigen	Host	Manufacturer	Dilution
human GPR56	Rabbit	Novus Biological	1/100
human CD44	Mouse	BD Pharmingen	1/50
mouse GPR56	Rabbit	Dr. Piao	1/250
mouse CD31	Rat	BD Pharmingen	1/200
mouse SOX2	Goat	Santa Cruz	1/400

3.8 Bioinformatic tools

3.8.1 Microarray data analysis

Microarray data were extracted with Agilent Feature Extraction Software version 10.7 and data analysis was carried out using GeneSpring GX version 11.5 (Agilent Technologies). In the hypoxic *versus* normoxic NSC study, normalized data was analyzed using paired t-test Limma (Morrissey and Diaz-Uriarte, 2009) to detect differentially expressed transcripts (multiple test correction: Benjamini & Hochberg). Principal Component Analysis (PCA) was performed with STATA software. Microarray data was also examined by Gene Set Enrichment Analysis (GSEA) using the KEGG pathway gene sets (for pathway enrichment) or the TFT gene sets (for TF binding site enrichment) (Subramanian et al., 2005). To correct for multiple hypothesis testing, GSEA uses FDR to focus on controlling the probability that each reported result is a false positive. Since the primary goal of GSEA is to generate hypothesis, an $FDR \leq 0.25$ is considered significant.

To obtain the *GPR56* knockdown-associated signature, normalized microarray data was analyzed using a paired two-class Significance Analysis of Microarrays (False Discovery Rate < 5%). Using the differentially expressed genes, a *GPR56* activity-associated signature was derived and applied onto the TCGA gene expression-based dataset.

3.9 Statistical analysis

Experimental data were represented as mean $\pm$ SEM (Standard Error of the Mean). Data represented with the help of the statistics software GraphPad Prism (GraphPad Prism v5.0a – GraphPad Software).

To compare two different groups, we used a Student's T test (paired or unpaired) for parametric variables. To compare different groups of samples, we used One-Way ANOVA (ANalysis of VAriance) test coupled to a Bonferroni post-test. Statistical significance for each analysis is shown in the corresponding figure.

Statistical significance for patient survival curves was analyzed with the help of the statistics software SPSS (IBM SPSS Statistics 20 – IBM) and the R Statisti-

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cal Software (The R Project for Statistical Computing). Statistical significance for each analysis is shown in the corresponding figure.

Results

4.1 Transcriptional profiling of hypoxic NSC identifies calcineurin-NFATc4 signaling as a major regulator of NSC biology

4.1.1 Long-term maintenance of mouse cortical E13.5 NSC in hypoxic conditions promotes self-renewal and proliferation

Culturing neural stem cells (NSC) at low oxygen levels has been shown to promote cell population growth, survival, proliferation, and multicompetency of NSC of different origins, such as rat and human mesencephalic NSC or mouse cortical NSC (Studer et al., 2000; Chen et al., 2007; Pistollato et al., 2007). One study reported enhanced clonogenicity at 5% oxygen of human NSC in adherent cultures (Pistollato et al., 2007). Although hypoxia enhances the expansion of immortalized human NSC cultured as neurospheres (Santilli et al., 2010), the effect of hypoxia on NSC self-renewal through a neurosphere formation assay had not been tested. We isolated mouse cortical NSC from E13.5 embryos, placed them immediately at either 5% or 20% O₂, and maintained them in these conditions for the whole length of the study (minimum of 10 days). We then measured self-renewal by measuring neurosphere formation ability at low density. Culturing NSC in hypoxic conditions dramatically increased the rate of

self-renewal (Figure 4.1A and 4.1B). Moreover, E13.5 mouse cortical NSC displayed increased proliferation at 5% as compared to 20% oxygen, as measured by EdU incorporation (Figure 4.1C). Therefore, physiologic hypoxia promotes cortical NSC self-renewal and proliferation.

4.1.2 Transcriptomic analysis of hypoxic NSC

Next, we set out to identify the molecular mechanisms mediating the effect of low oxygen levels on NSC biology. We profiled the global effect of physiologic hypoxia on NSC gene expression using an unbiased approach by genome-wide microarray analysis of NSC cultured at 5% or 20% O₂ (Figure 4.2A). Unsupervised principal component analysis (PCA) of microarray data showed a reasonable separation between the hypoxia and normoxia groups (Figure 4.2B). PCA also showed some separation of the samples based on mouse donor. These data indicate that NSC from an individual mouse mostly maintain their identity regardless of oxygen levels compared to NSC from different mice, which contributes to gene expression variability among samples. However, low oxygen conditions still induce a significant shift in gene expression among NSC from different animals, thus showing that our microarray results are consistent and robust.

Comparative analysis of the transcriptomes revealed 374 significantly deregulated protein-coding genes. 64 of these genes displayed expression changes larger than four-fold in hypoxia with respect to normoxia (59 upregulated and 5 downregulated genes) (Table 4.1). Microarray expression changes were validated using RT-qPCR for a subset of genes of interest (Figure 4.2C). Notably, the NADPH dehydrogenase [ubiquinone] 1 alpha complex, 4-like 2 (*Ndufa4l2*) gene was strongly induced by hypoxia, as previously reported (Tello et al., 2011). Upregulation of this gene is part of the cell's metabolic adaptation to hypoxia. We also observed an increase in expression of the subunit alpha 3 of the gamma-aminobutyric acid (GABA) A receptor (*Gabra3*). Interestingly, GABAA receptors are involved in the control of stem cell behavior by negatively regulating ESC and neural crest stem cell proliferation (Andäng et al., 2008). We also determined by RT-qPCR the upregulation of two well-described players in the cell's response to hypoxia, hypoxia inducible factor 3 (*Hif3a*), member of the hypoxia inducible factor family of transcription factors, and vascular endothelial growth factor (*Vegf*), a potent pro-angiogenic factor. These results suggest that

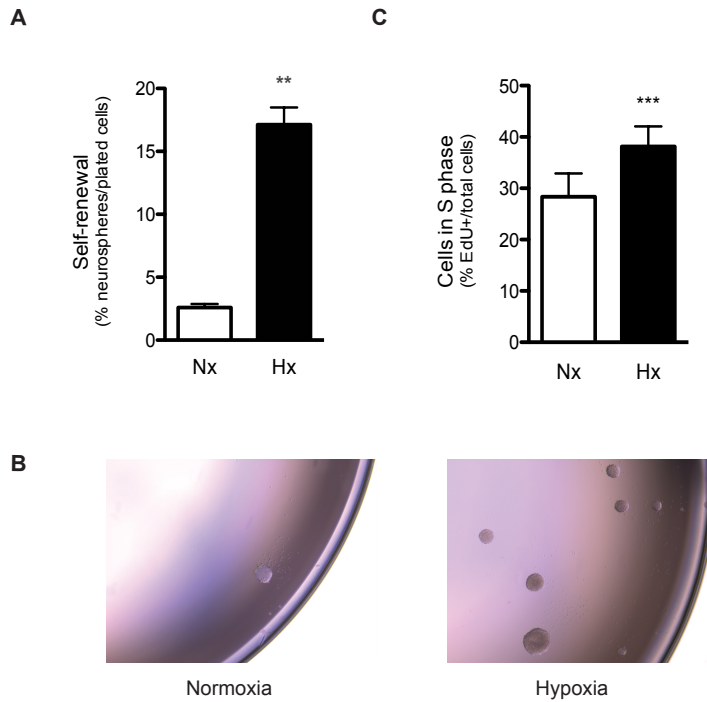


Figure 4.1: *Physiologic hypoxia promotes NSC self-renewal and proliferation.*

(A) Self-renewal of mouse E13.5 NSC maintained in 5% oxygen (hypoxia) or 20% oxygen (normoxia) was measured as the efficiency of single cells to give rise to neurospheres at low density. Data are represented as mean $\pm$ SEM ($n = 4$, paired t-test, ** $p < 0.005$). (B) Representative images of neurospheres in the self-renewal assay described in A. (C) Cell proliferation of NSC maintained in hypoxia or normoxia as in A was assessed by EdU incorporation. Data are represented as mean $\pm$ SEM ($n = 8$, paired t-test, *** $p < 0.001$).

low oxygen levels induce changes in the transcriptome of NSC that might affect important NSC functions such as metabolism and proliferation.

4.1.3 Signaling pathways preferentially activated under physiologic hypoxic conditions: calcium signaling pathway

Some differences in gene expression may be subtle but still biologically significant if they involve a coordinated change at the level of signaling pathways or co-regulated gene sets rather than individual genes. This type of changes might remain unnoticed when using a classical statistical analysis of microarray data. To gain more insight into the biological significance of our results, we applied Gene Set Enrichment Analysis (GSEA) to evaluate microarray data at the level of gene sets. This approach was aimed at identifying pathways and transcription factors (TF) that are activated in NSC cultured under oxygen concentrations that more closely resemble their normal tissue microenvironment. We interrogated for pathway enrichment by using the gene sets from the KEGG pathway database.

On one hand, we observed pathways enriched in normoxic NSC included pathways classically involved in cell proliferation, including basal TF, cell cycle and DNA replication (Figure 4.3A). However, we have observed that NSC in hypoxic conditions have a higher rate of cell proliferation compared to normoxic cells (Figure 4.1C). These apparently paradoxical results suggest that pathways controlling proliferation in hypoxic NSC might be different from the ones regulating normoxic NSC proliferation.

On the other hand, GSEA-KEGG pathway analysis revealed an enrichment of genes within both the glycolytic and glycogen synthesis pathways in hypoxic NSC (Figure 4.3B). These results are consistent with the described metabolic adaptation of cells to hypoxia. During hypoxia, cells undergo an anaerobic switch that lowers mitochondrial oxidative phosphorylation activity, thereby reducing oxygen consumption. At the same time, there is an increase in glucose uptake and the rate of glycolysis (Iyer et al., 1998; Pescador et al., 2010; Papandreou et al., 2006; Aragonés et al., 2008). Accordingly, as described above, the gene that showed a larger fold increase in hypoxia was the mitochondrial *Ndufa4l2* (Table 4.1). This protein has been shown to reduce mitochondrial

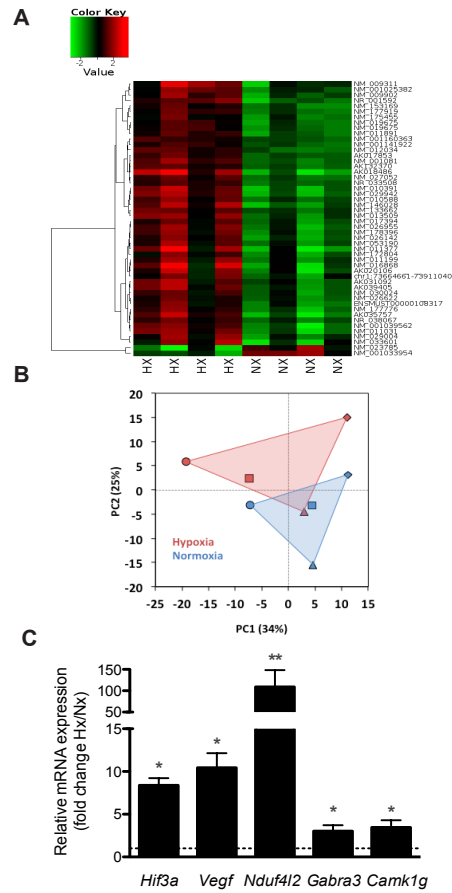


Figure 4.2: *Genome-wide expression microarray analysis of hypoxic versus normoxic NSC.*

(A) RNA was isolated from adherent NSC maintained in hypoxia or normoxia and hybridized to a gene expression microarray chip. Shown is the heatmap of the top 50 most significantly upregulated and downregulated genes ($n = 4$, paired t-test limma). (B) Unsupervised PCA of microarray datasets. Circle, square and diamonds represent NSC isolated from different mice. (C) RT-qPCR of selected genes identified by microarray analysis. Shown is the fold change of mRNA expression in hypoxia with respect to normoxia. Data are represented as mean $\pm$ SEM ($n = 3$ -4, paired t-test, * $p < 0.05$, ** $p < 0.005$). See also Table 4.1.

Results

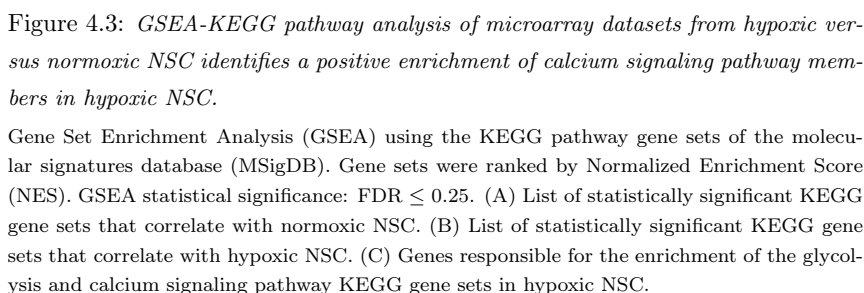
Probe ID	Gene name	Fold Change (Hx vs Nx)	Upregulation or downregulation (Hx vs Nx)	FDR_indep
A_55_P1971244	Ndufa4l2	36.7	up	0.032
A_55_P2092750	Car9	19.0	up	0.030
A_55_P2190426	Sim2	11.3	up	0.021
A_51_P155873	Ppp1r3g	10.2	up	0.028
A_55_P2048759	Mgap	9.0	up	0.044
A_51_P480119	Prelid2	8.8	up	0.024
A_51_P128320	Muc2	8.8	up	0.040
A_55_P2005735	Trpm2	8.7	up	0.026
A_55_P2099363	Stac2	8.6	up	0.021
A_55_P2037343	H2-Q10	8.5	up	0.024
A_52_P663413	P4ha2	7.5	up	0.021
A_66_P120736	Brip3	7.5	up	0.031
A_55_P2015541	Hif3a	7.4	up	0.024
A_51_P405606	Ndrp1	7.2	up	0.043
A_52_P319438	Ankrd37	7.1	up	0.024
A_52_P579531	Pdlim3	6.9	up	0.028
A_51_P431046	St8sia3	6.6	up	0.050
A_55_P2000284	Phox2a	6.5	up	0.029
A_51_P406429	Pdk1	6.4	up	0.044
A_55_P2176065	Lrrc9	6.3	up	0.040
A_55_P2066116	Bcl3	5.7	up	0.021
A_55_P2086128	Fa2h	5.7	up	0.044
A_51_P267494	Cdc42ep3	5.6	up	0.039
A_55_P2019457	Eno2	5.6	up	0.024
A_55_P1954693	Tac1	5.5	up	0.024
A_55_P1961014	Selenbp1	5.5	up	0.040
A_55_P2000354	Rgs11	5.4	up	0.024
A_51_P220934	Vstm5	5.3	up	0.021
A_55_P2109326	Ache	5.3	up	0.031
A_55_P1984655	Smtnl2	5.0	up	0.024
A_52_P354744	Slc2a3	5.0	up	0.033
A_55_P2083794	Tmem159	5.0	up	0.028
A_55_P2005730	Trpm2	5.0	up	0.024
A_55_P2019312	Car12	4.8	up	0.024
A_51_P213359	Has2	4.8	up	0.044
A_51_P448971	Sncb	4.7	up	0.038
A_51_P258721	Tpsg1	4.6	up	0.024
A_52_P301261	C3HC4	4.6	up	0.047
A_52_P249424	Vegfa	4.5	up	0.039
A_55_P2109382	Adora2a	4.5	up	0.027
A_55_P2034245	Meox1	4.4	up	0.033
A_55_P2183473	Cldn3	4.4	up	0.024
A_52_P66226	Rab33a	4.4	up	0.028
A_55_P2138714	Sgcd	4.4	up	0.024
A_55_P2031721	Npas1	4.4	up	0.030
A_51_P207751	Guca1a	4.3	up	0.044
A_55_P2036693	Cd40	4.3	up	0.040
A_52_P48569	Slc38a4	4.3	up	0.024
A_55_P2117614	Tnfrsf13c	4.3	up	0.030
A_55_P2071726	Rasgef1c	4.2	up	0.024
A_55_P1963463	Gabra3	4.2	up	0.033
A_51_P451338	Jag2	4.2	up	0.024
A_55_P2017054	Ighv6-4	4.2	up	0.048
A_66_P104980	Tceal5	4.2	up	0.024
A_52_P238468	Gipr	4.1	up	0.047
A_55_P2038822	Jph4	4.1	up	0.024
A_52_P638895	Vegfa	4.1	up	0.046
A_55_P2109327	Ache	4.0	up	0.035
A_51_P428372	Pbbp	-6.0	down	0.024
A_55_P2139942	Calca	-5.0	down	0.029
A_52_P154101	Calca	-4.9	down	0.021
A_52_P145415	Ptch2	-4.3	down	0.043
A_55_P1958798	Mobp	-4.2	down	0.028

Table 4.1: *NSC genes differentially expressed in hypoxia versus normoxia.*

Shown are the genes significantly up- or downregulated by more than four-fold in NSC maintained at 5% oxygen with respect to 20% oxygen (n = 4, paired t-test limma, multiple test correction: Benjamini & Hochberg, statistical significance: FDR < 0.05).

oxygen consumption by inhibiting Complex I activity, which limits the intracellular ROS production under hypoxic conditions (Tello et al., 2011). Therefore, the naturally hypoxic microenvironment of NSC might physiologically support a non-oxidative metabolism in these cells. Remarkably, several members of the aldehyde dehydrogenase family (*Aldh9a1*, *Aldh1a3*, *Aldh1b1*, *Aldh7a1* and *Aldh2*) were part of the leading-edge subset of the glycolysis gene set (Figure 4.3C). Indeed, *Aldh9a1* is the most significantly upregulated gene within the glycolytic pathway (Figure 4.3C). Interestingly, the ALDH family of enzymes is enriched in different types of stem and cancer stem-like cells (Corti et al., 2006; Bar et al., 2007; Armstrong et al., 2004), supporting the notion that a hypoxic environment might favor a stem cell phenotype. Taken together, these results suggest that the naturally hypoxic microenvironment of NSC might physiologically support a non-oxidative metabolism that promotes stemness.

Interestingly, GSEA-KEGG pathway analysis identified a positive enrichment of calcium signaling pathway members in NSC cultured at 5% oxygen, suggesting that this pathway is activated under physiologic hypoxic conditions (Figure 4.3B). The calcium signaling pathway enrichment in response to hypoxia involved an increase in the expression of calcium signaling mediators, including *Camk1g* (calcium/calmodulin-dependent protein kinase 1 gamma) and *Calml3* (calmodulin-like 3), which might potentiate the response to a given calcium concentration within the cell. *Camk1g* is one of the genes validated by RT-qPCR to be upregulated by physiologic hypoxia (Figure 4.2C). At the same time, we observed an increase in the expression of different calcium channels, including *Ryr1* (ryanodine receptor 1), *Cacna1c*, *Cacna1d* and *Cacna1h* (L-type voltage-dependent calcium channels, subunits alpha 1C, 1D and 1H, respectively), which might promote an increase in the intracellular calcium concentration (Figure 4.3C). Both effects might contribute to an increase in calcium signaling in hypoxic conditions. It has been described that calcium transient-driven purinergic signaling in neural progenitor cells induces their proliferation (Lin et al., 2007). Therefore, an increase in calcium signaling in hypoxic conditions might promote NSC functions, including proliferation.



4.1.4 Identification of transcription factors activated under physiologic hypoxic conditions: NFATc4

The primary goal of this study was to identify the TF that orchestrate the NSC response to hypoxia. To this end, we performed Gene Set Enrichment Analysis using the transcription factor target gene sets (TFT). These gene sets are defined as groups of genes that share a transcription factor binding site according to the TRANSFAC database (version 7.4, <http://www.gene-regulation.com/>). GSEA-TFT revealed that the gene targets of the E2F transcription factor were the most significantly enriched in normoxic NSC, thereby suggesting that E2F was predominantly activated under normoxic conditions (Figure 4.4A). E2F is a transcription factor that positively regulates the cell cycle (Müller and Helein, 2000). Therefore, this result was in agreement with the GSEA-KEGG, which identified an enrichment of cell replication-related pathways in normoxia (Figure 4.3A), thereby reinforcing the idea that TF and pathways controlling normoxic NSC proliferation may be different from the ones regulating hypoxic NSC proliferation.

Conversely, we found that more than 110 TFT gene sets were enriched in hypoxic NSC, which suggests that TF controlling expression of these genes might be activated in these conditions. Such TF included E12 (transcription factor 12), GATA (GATA transcription factor) and AP-1 (activator protein 1) (Figure 4.4B). As expected, HIF1 binding sites were enriched in genes differentially expressed in hypoxic NSC *versus* normoxic NSC (V\$HIF1_Q5, NES = 1.33, FDR = 0.22). We also identified other TF previously described to be activated by hypoxia in other cell types, such as NF κ -B (nuclear factor κ -light-chain-enhancer of activated B-cells) (Figure 4.4B) (Rius et al., 2008). Importantly, there are a large number of TFT gene sets that have a higher normalized enrichment score than HIF1, thus highlighting their potential role as important regulators of the hypoxic response. These results show that the hypoxic transcriptional response goes far beyond the sole activation of the HIF family of TF. The transcription factor motif gene set with the highest normalized enrichment score (NES) was V\$E12_Q6, indicating this is the gene set that better correlates with hypoxia. Strikingly, E12 (E47/Tcf3) is a TF that is used to reprogram fibroblasts into induced NSC (iNSC) (Han et al., 2012). These results robustly validate our transcriptional profiling approach to identify factors that are important in NSC biology by searching for TFT enriched in hypoxic NSC.

Remarkably, in several of the top twenty TFT gene sets from our GSEA-TFT (E12, AP-1, CP2 and STAT5A), the transcription factor *Nfatc4* was the gene with the highest NES, indicating that *Nfatc4* was the gene that better correlated with the hypoxic phenotype in these gene sets (Figure 4.4C). Accordingly, RT-qPCR and western blot analysis revealed that *Nfatc4* mRNA and protein levels were upregulated in hypoxic NSC (Figure 4.4D and Figure 4.5A). Upregulation of *Nfatc4* in hypoxic conditions was also observed in our microarray analysis, although it was not statistically significant (data not shown). In addition, the NFATc4 TFT gene set (V\$NFAT_Q6) was significantly enriched in hypoxic NSC (NES=1.30, FDR=0.25), indicating that NFATc4 targets were also upregulated by hypoxia (Figure 4.4F). Upregulation of three of the NFATc4 target genes, *Trim47*, *FoxP2* and *Id2*, was validated by RT-qPCR (Figure 4.4E). Expression of the rest of NFAT family members (*Nfatc1* to *Nfatc3*) didn't change between normoxia and hypoxia as assessed by microarray analysis and RT-qPCR (Figure 4.4D). The latter results were corroborated by our GSEA-TFT analysis, where neither *Nfatc1*, nor *Nfatc2*, nor *Nfatc3* TFT gene sets showed up as significantly enriched in hypoxic NSC (data not shown).

The above results suggest that *Nfatc4* expression is regulated at the transcriptional level by oxygen concentrations. HIF-1 regulation by hypoxia depends on both protein stabilization and transcriptional activation (Rius et al., 2008; Semenza, 2001). In the presence of high oxygen levels HIF-1 is ubiquitinated and subjected to proteasomal degradation. Under reduced oxygen availability, the fraction of HIF1 that is ubiquitinated decreases dramatically, resulting in an accumulation of the protein and, consequently, promoting the activation of different target genes (Jiang et al., 1996). Since oxygen availability controls HIF-1 activity at the protein level, we asked whether NFATc4 was also regulated via this mechanism. To test this hypothesis, NSC maintained under normoxia were incubated at 5% oxygen during different periods of time. Western blot analysis revealed that NFATc4 protein levels remained unaltered at short time points after the switch to hypoxia. On the contrary, NFATc4 protein was only upregulated after long term exposure to hypoxia (Figure 4.5B), which would better correlate with the activation of gene transcription rather than an increase in protein stability. To further confirm that NFATc4 protein was not regulated by proteasomal degradation, we treated normoxic cells with the proteasome inhibitor MG132. Inhibition of the proteasome didn't affect NFATc4 protein levels, thereby suggesting that oxygen levels are not regulating *Nfatc4* levels by

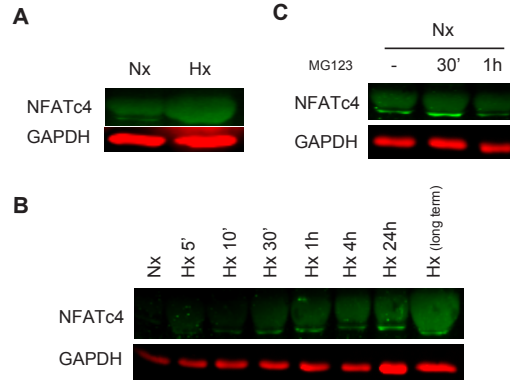


Figure 4.5: *Nfatc4* levels in hypoxic NSC are not regulated by the proteasome.

(A) Western Blot (WB) analysis of NFATc4 in NSC maintained long-term in normoxia or hypoxia. (B) WB analysis of NFATc4 in NSC maintained in normoxia and exposed to hypoxia (5% oxygen) for different periods of time. (C) Normoxic NSC were pre-treated with the proteasome inhibitor MG132 or vehicle control (DMSO) for 1 hour and collected after additional 30 minutes or 1 hour. After the indicated times, cells were harvested and lysates subjected to WB analysis of NFATc4 protein.

controlling protein stability (Figure 4.5C). Taken together, these results suggest that *Nfatc4* is upregulated in hypoxic NSC by transcriptional activation and point to NFATc4 as a good candidate to regulate NSC functions.

4.1.5 Functional characterization of calcineurin-NFATc4 signaling in NSC proliferation and self-renewal

Calcium signaling is known to affect the activity of several transcription factors, including the NFAT family of transcription factors. Cytoplasmic NFATs (NFATc1 through NFATc4) are activated by calcineurin, a calcium/calmodulin dependent serine phosphatase. Upon dephosphorylation by calcineurin, NFATs shuttle to the nucleus and bind to their target promoters (Crabtree and Olson, 2002; Hogan et al., 2003). In agreement with our GSEA-TFT results regarding NFATc4 (Figure 4.4), the GSEA-KEGG pathway analysis identified a positive enrichment of calcium signaling pathway members in NSC cultured at 5% oxygen, suggesting that this pathway is activated in hypoxic NSC (Figure 4.3B). Therefore, we hypothesized that calcium-calcineurin-NFAT signaling might be

activated under physiologic hypoxic conditions and have a relevant role in NSC functions.

To test our hypothesis, we functionally characterized the role of calcineurin-NFAT signaling in the proliferation and self-renewal of hypoxic NSC. Using the pharmacological calcineurin inhibitors cyclosporin A (CsA) and FK-506 (tacrolimus), we inhibited NFAT-dependent transcriptional activity in a dose-dependent manner (Figure 4.6A). Importantly, CsA plus FK-506 decreased both self-renewal and proliferation of hypoxic NSC, thus suggesting that calcineurin-NFAT signaling promotes NSC proliferation and self-renewal (Figure 4.6B and 4.6C).

Next, we set out to identify what NFAT family member might be controlling NSC properties downstream of calcineurin. We have previously observed that *Nfatc4* was the only NFAT family member upregulated in hypoxia (Figure 4.4D). Nevertheless, we sought to analyze the subcellular localization of the four NFAT family members that are regulated by calcium (Figure 4.7A). The subcellular localization of NFATc1 (NFAT2) was cytoplasmic. NFATc2 (NFAT1) was expressed at very low levels and was mainly present in the cytoplasm, although it would also faintly stain the nuclei of some cells. NFATc3 (NFAT4) displayed both nuclear and cytoplasmic localization. NFATc4 (NFAT3) was expressed at high levels and was heavily concentrated in the nuclei of hypoxic NSC. The specificity of the antibody was tested by peptide competition assay (Figure 4.7B). Therefore, its nuclear localization, together with its upregulation in hypoxia and the GSEA-TFT results, point to NFATc4 as the NFAT family member that might mostly contribute to NSC properties under physiologic hypoxia.

To test whether NFATc4 is functionally relevant in the control of NSC functions, we performed *Nfatc4* silencing using two different *Nfatc4* siRNA and assessed its effects on NSC self-renewal. *Nfatc4* siRNA #1 achieved a 50% knockdown efficiency, while *Nfatc4* siRNA #2 induced an 80% reduction in *Nfatc4* mRNA expression (Figure 4.7C). Accordingly, some of the NFATc4 gene targets were also reduced in *Nfatc4* siRNA-transfected cells. *FoxP2* and *Id2* were two of the NFATc4 target genes most robustly and significantly downregulated, specially by siRNA #2 (Figure 4.7D). Importantly, *Nfatc4* siRNA #2-transfected NSC displayed a significant reduction in self-renewal (Figure 4.7E). Therefore, NFATc4 is the NFAT family member that most probably mediates the effect of calcineurin on NSC self-renewal.

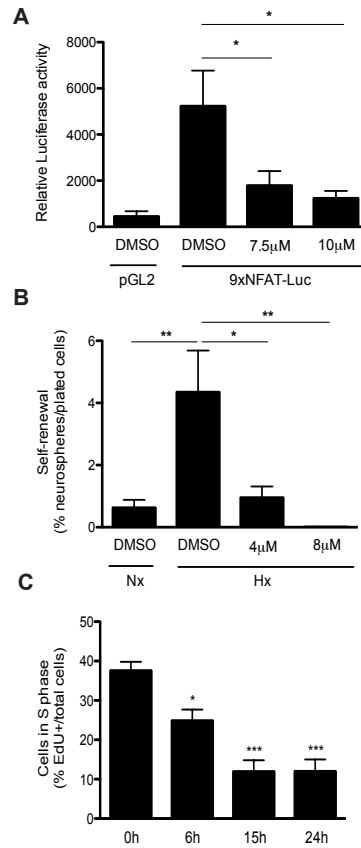


Figure 4.6: *Calcineurin promotes NSC self-renewal and proliferation.*

(A) NSC were electroporated with a luciferase reporter gene controlled by 9 NFAT-binding sites, together with a renilla expression plasmid to serve as an internal control, and treated with increasing concentrations of the calcineurin inhibitors cyclosporin A (CsA) and FK-506 (tacrolimus) or vehicle control (DMSO). After O/N drug treatment, cells were subjected to a dual luciferase assay. Data are represented as mean $\pm$ SEM ($n = 3$, ANOVA, $*p < 0.05$). (B) NSC self-renewal was assessed by low density neurosphere formation assay in the presence of different concentrations of CsA + FK-506 or vehicle control. Data are represented as mean $\pm$ SEM ($n = 3$, ANOVA, $*p < 0.05$, $**p < 0.01$). (C) NSC maintained in hypoxia were treated with either CsA + FK-506 (7.5 μ M) or vehicle control. Cell proliferation was assessed by EdU incorporation at the indicated time points after drug treatment. Data are represented as mean $\pm$ SEM ($n = 3$, ANOVA, $*p < 0.05$, $***p < 0.005$).

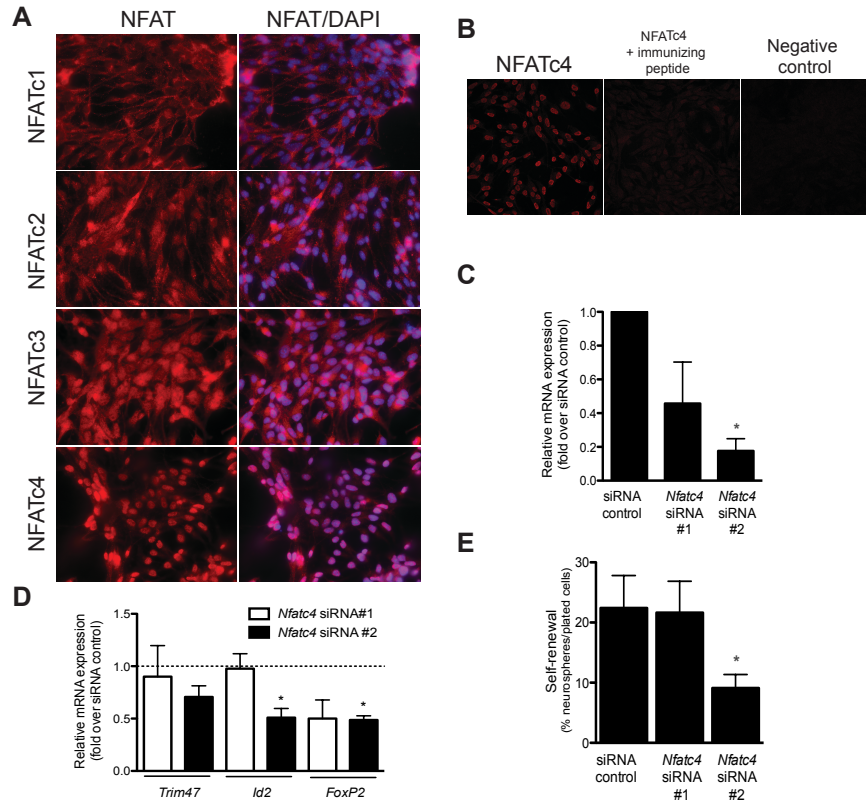


Figure 4.7: *NFATc4* promotes NSC self-renewal in vitro.

(A) Immunocytochemistry of calcium regulated NFAT family members. NSC maintained in hypoxia were stained with antibodies against NFATc1 through NFATc4. Nuclei were stained with a DNA dye (DAPI). (B) Immunocytochemistry of NFATc4 in hypoxic NSC was performed using either no primary antibody as a negative control, an NFATc4 antibody previously incubated with an NFATc4 immunizing peptide or an NFATc4 antibody alone. Nuclei were stained with a DAPI. (C) *Nfatc4* mRNA expression was assessed by RT-qPCR of hypoxic NSC electroporated with two different siRNAs directed against *Nfatc4* or a control siRNA. Data are represented as mean $\pm$ SEM ($n = 3$, paired t-test, $*p < 0.05$). (D) NFATc4 target gene expression in *Nfatc4*-knockdown NSC was assessed by RT-qPCR. Data are represented as mean $\pm$ SEM ($n = 3$, paired t-test, $*p < 0.05$). (E) NSC maintained in hypoxia were electroporated with a siRNA directed against *Nfatc4* or a control siRNA. Viable cells were immediately sorted and plated at low density. Neurosphere formation was assessed 7 days after plating. Data are represented as mean $\pm$ SEM ($n = 3$, paired t-test, $*p < 0.05$).

Then, we further tested NFATc4 function *in vivo*. First we performed immuno-histochemistry of NFATc4 in the brain of E15 mouse embryos. We observed that NFATc4 was also expressed in the nuclei of cells in the ventricular zone where neural progenitors reside (Figure 4.8A). To characterize the NFATc4 function *in vivo*, we used *in utero* electroporation of *Nfatc4* siRNA #2 in E13.5 mouse embryos. To analyze the effect of *Nfatc4* knockdown on neural progenitor proliferation, we measured the number of neural progenitors in the S-phase of the cell cycle. Control siRNA-electroporated neural progenitors displayed a higher proliferative rate than *Nfatc4* siRNA-electroporated cells (Figure 4.8B). Furthermore, self-renewal of neural progenitors was markedly reduced by knockdown of *Nfatc4* in mouse embryos since the percentage of neural progenitors that re-enter the cell cycle was significantly higher in siRNA control-electroporated progenitors than in *Nfatc4* siRNA-electroporated cells (Figure 4.8C). These results are in agreement with our results in cultured NSC, thus indicating that NFATc4 controls NSC proliferation and self-renewal *in vitro* and *in vivo*.

Taken together, our results suggest that the calcineurin-NFATc4 axis plays a major role in the regulation of NSC biology under physiologic low oxygen concentrations. We have also found that *Id2* is the NFATc4 target whose downregulation by NFATc4 siRNA, better correlates with their effects on self-renewal. Given the role of ID2 in preserving the neural stem cell state and preventing neuronal differentiation by antagonizing proneural and Olig basic helix-loop-helix transcription factors, we speculate that ID2 might be a major mediator of the effect of NFATc4 on NSC self-renewal (Ross et al., 2003; Iavarone and Lasorella, 2006). Therefore, we hypothesize that NFATc4 might control NSC proliferation and self-renewal by promoting the expression of transcription factors, including ID2, that contribute to the maintenance of the stem cell state.

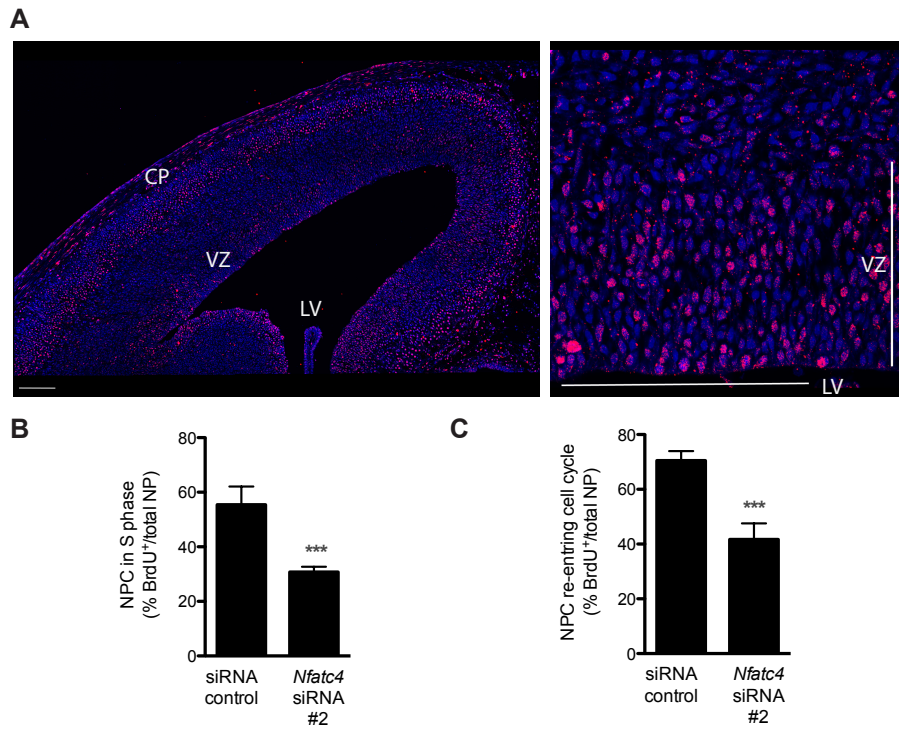


Figure 4.8: *NFATc4* promotes NSC self-renewal and proliferation in vivo.

(A) Immunohistochemistry of NFATc4 in the brain of E15 mouse embryos. Left panel, coronal section of the mouse brain at low magnification. Shown is one of the cerebral hemispheres including the lateral ventricle (LV), ventricular zone (VZ) and cortical plate (CP). Scale bar: 100 μ m. Right panel, higher magnification confocal image of the immunostaining shown in left panel focusing on the VZ. Scale bar: 100 μ m. (B) Proliferation was assessed by counting the number of neural progenitors (NP) in S-phase by BrdU incorporation 30 minutes before sacrifice. Data are represented as mean $\pm$ SEM (n = 3, Chi square test, ***p < 0.001). (C) Self-renewal was assessed by counting the number of neural progenitors (NPC) re-entering the cell cycle by BrdU incorporation 24h before sacrifice. Data are represented as mean $\pm$ SEM (n = 3, Chi square test, ***p < 0.001).

4.2 GPR56 is a NSC factor that restricts the proneural to mesenchymal transition in GBM by inhibiting the NF- κ B pathway

4.2.1 *GPR56* is progressively upregulated during ESC specification to NSC

Every GBM molecular subtype holds different types of oncogenic mutations affecting different major signaling pathways (Verhaak et al., 2010). This might suggest that every subtype is independently driven by these distinct alterations. However, there is an inherent plasticity of GIC to transition from one subtype to another, specially from a PN to a MES subtype (Bhat et al., 2013). The different GBM subtypes might represent different stages in tumor evolution, with a PN-like tumor being the starting point and the MES subtype being one of the most advanced stages (Ozawa et al., 2014). Therefore, we hypothesized that during GBM tumor progression GIC would lose phenotypic traits of the neural lineage while acquiring properties of less committed stem cells.

In order to find novel regulators of GBM progression from a PN to a MES subtype, we searched for factors differentially expressed in NSC *versus* less committed stem cells, such as embryonic stem cells (ESC). GPR56 is an adhesion GPCR highly expressed in neural NSC, so we interrogated whether this receptor would fit our criteria (Bai et al., 2009; Piao et al., 2004; Li et al., 2008). We compared *GPR56* mRNA expression between NSC and two non-neural stem cell types: ESC, representative of a non-committed stem cell type, and mesenchymal stem cells (MSC), representative of a stem cell with a mesenchymal phenotype. RT-qPCR analysis revealed that both MSC and ESC expressed negligible levels of *GPR56* whereas NSC express high levels of *GPR56* mRNA in both human and mouse cells (Figure 4.9A and 4.9B). We then analyzed *GPR56* mRNA expression during neural differentiation of human ESC. Interestingly, *GPR56* was poorly expressed in H9 human ESC but highly upregulated when ESC undergo differentiation into the neural lineage (Figure 4.9C). Our results show that *GPR56* is highly enriched in stem cells of the neural lineage as opposed to other non-neural stem cells, including less committed ESC.

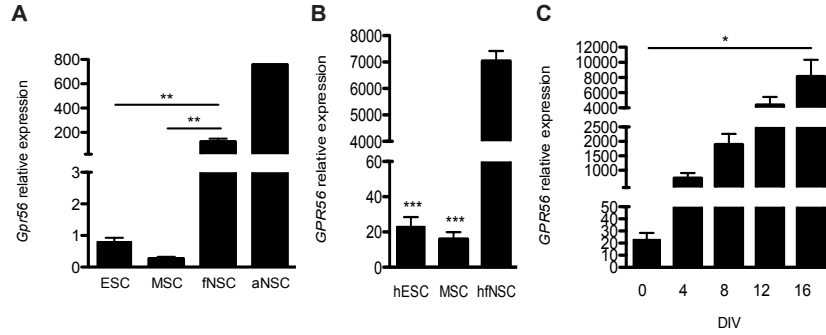


Figure 4.9: *GPR56* is progressively upregulated during ESC specification to NSC.

(A) *GPR56* mRNA expression was analyzed by RT-qPCR in mouse ESC, MSC, fetal NSC and adult NSC. Data are represented as mean $\pm$ SEM of 3 independent experiments (t-test, $**p < 0.01$). (B) *GPR56* mRNA expression was analyzed by RT-qPCR in human ESC, MSC and fetal NSC. Data are represented as mean $\pm$ SEM of 8 (ESC), 5 (MSC) or 2 (NSC) independent experiments (t-test, $***p < 0.005$). (C) H9 human ESC cell line was differentiated along the neural lineage. *GPR56* mRNA expression was analyzed at the indicated time points by RT-qPCR. Data are represented as mean $\pm$ SEM of 3 independent experiments (t-test, $*p < 0.05$).

4.2.2 *GPR56* is enriched in PN-subtype GBM and inversely correlated with MES markers

MES-subtype GBM are believed to evolve from a proneural-like malignant lesion (Ozawa et al., 2014). Since *GPR56* is poorly expressed in MSC or ESC and upregulated during the neural specification of ESC, we asked whether *GPR56* would be involved in the progression of GBM from a proneural-like tumor to a MES-subtype GBM. First, we analyzed *GPR56* mRNA expression in GBM specimens from The Cancer Genome Atlas (TCGA) studies. Strikingly, group comparison analysis between the four different GBM subtypes revealed a significant enrichment of *GPR56* in PN *versus* MES GBM (Figure 4.10A). In addition, *GPR56* expression positively correlated with well-established PN signature genes: *OLIG2* (oligodendrocyte transcription factor 2; Spearman's rank correlation coefficient (SCC) = 0.60), *SOX2* (sex determining region-box 2; SCC = 0.59), *SOX9* (sex determining region-box 9; SCC = 0.64), *NCAM1* (neural cell adhesion molecule 1; SCC = 0.54) and *ASCL1* (achaete-scute complex homolog 1; SCC = 0.54), while it negatively correlated with MES genes: *TGFBR1* (transforming growth factor, beta receptor 1; SCC = -0.42), *TGFBR2* (trans-

forming growth factor, beta receptor 2; $SCC = -0.32$) and *TGFBI* (transforming growth factor, beta-induced; $SCC = -0.39$) (extracted from cBioPortal for Cancer Genomics, www.cbioportal.org/). Furthermore, *GPR56* was co-expressed with wild type *NF1* (neurofibromatosis 1; $SCC = 0.52$), a tumor suppressor gene that is frequently mutated in MES-subtype GBM (Figure 4.10B).

To validate the above results in another set of human GBM, we analyzed GPR56 expression by immunohistochemistry in GBM specimens from Hospital Clínic de Barcelona (HCB). Importantly, GBM tumors with highest expression of GPR56 displayed very low levels of CD44, a well-established MES marker. Conversely, the majority of tumors with very low expression of GPR56 were highly immunoreactive for CD44 (Figure 4.10C). These results are in agreement with our previous analysis of the TCGA dataset. Taken together, our results suggest that GPR56 might be downregulated during the mesenchymal transition of PN-subtype GBM.

4.2.3 *GPR56* is highly expressed in PN GIC and down-regulated during their MES transition in response to $TNF-\alpha$

We next analyzed whether glioma stem-like initiating cells (GIC) show similar patterns of *GPR56* expression as whole tumors. To determine the association of our cultured GIC with either the PN or MES subtype, we characterized them using a well-established set of three PN (*OLIG2*, *SOX2* and *SOX9*) and four MES (*FN1* (fibronectin 1), *CHI3L1* (chitinase-3-like protein 1), *CD44* (cluster of differentiation 44) and *CTGF* (connective tissue growth factor)) genes. Most of our GIC displayed PN characteristics (Figure 4.11A), according to a previous study that described loss of mesenchymal features in isolated GIC in culture (Bhat et al., 2013). We then selected four MES and four PN GIC and measured *GPR56* and *CD44* mRNA expression by RT-qPCR. *GPR56* levels were very low in most MES GIC, whereas PN GIC expressed higher levels of *GPR56* (Figure 4.11B). Similar results were obtained when measuring GPR56 protein levels by immunoblotting (Figure 4.11C). It should be noted that although *GPR56* mRNA levels were high in two of the MES cell lines (GSC2 and GSC28), GPR56 protein levels were significantly lower than in the rest of PN GIC (Figure 4.11C). Additionally, we validated these results in another set of thirty-one different GIC lines (Ohio State University (OSU) dataset). Microarray analysis showed that

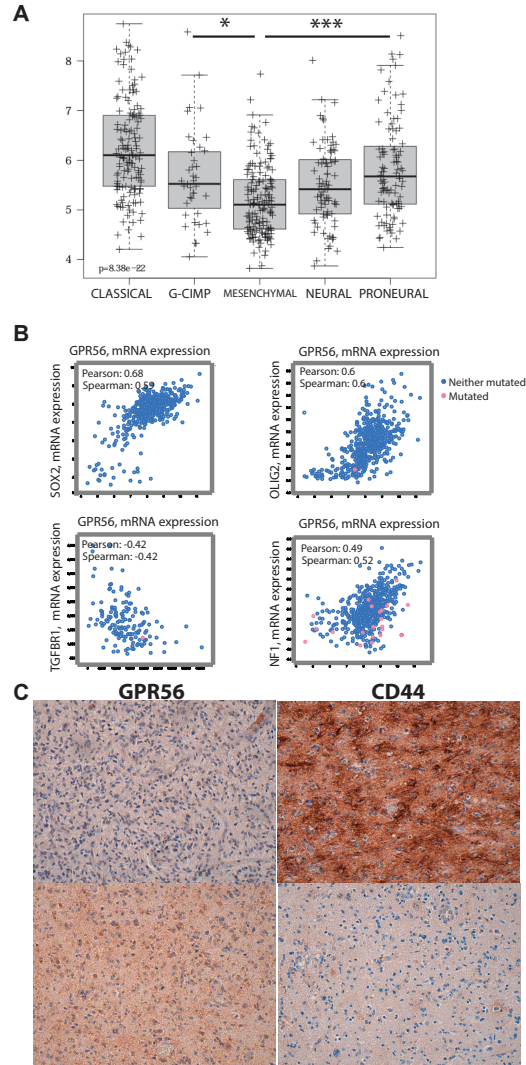


Figure 4.10: *GPR56* is enriched in PN-subtype GBM and inversely correlated with MES signature markers.

(A) *GPR56* mRNA expression across the different subtypes of GBM was retrieved from the TCGA dataset (Affimetrix U133A platform). Box-plot diagram showing the *GPR56* expression levels in patients with GBM (ANOVA, * $p < 0.05$, *** $p < 0.001$). (B) Correlation of *GPR56* mRNA expression with the PN markers *SOX2* and *OLIG2*, and the MES marker *TGFBR1*, and the tumor suppressor *NF1* (TCGA dataset). Correlation plots were obtained from the cBioPortal for Cancer Genomics. (C) Immunohistochemistry of *GPR56* and *CD44* in paraffin-embedded human GBM specimens from the Hospital Clínic de Barcelona dataset (20x magnification).

almost all PN GIC express higher *GPR56* mRNA levels compared to MES GIC (Figure 4.11D). Interestingly, the three PN GIC lines with low *GPR56* expression also displayed some MES features (data not shown).

To further study the differential expression of *GPR56* in PN *versus* MES GIC, we sorted GIC cell lines into CD44^{high} and CD44^{low} subpopulations. Since CD44 is enriched in cells with mesenchymal properties, we considered CD44^{high} cells as a model for the mesenchymal subtype, while CD44^{low} cells are considered to be non-mesenchymal. We next analyzed the expression levels of *GPR56* in CD44^{high} and CD44^{low} cells. RT-qPCR and western blot analysis revealed that *GPR56* is poorly expressed in CD44^{high} whereas it is highly enriched in CD44^{low} GIC (Figure 4.12A and 4.12B).

Given the loss of MES signature in cell cultured-GIC isolated from MES GBM, it has been suggested that specific factors in the tumor microenvironment might be required to induce a MES phenotype. The pro-inflammatory cytokine TNF- α (tumor necrosis factor alpha) has been pointed out as a major inducer of MES features in GIC (Bhat et al., 2013). TNF- α treatment of our GIC lines resulted in an increase in their MES signature (Figure 4.13A). However, TNF- α was not sufficient to induce a global reduction in PN genes, as previously described (Figure 4.13A) (Bhat et al., 2013). Since *GPR56* is poorly expressed in MES GIC, we next asked whether TNF- α treatment would downregulate *GPR56* expression in PN GIC. We selected two of the best TNF- α -responders and, interestingly, we observed that TNF- α induces a decrease in *GPR56* mRNA expression in CD44^{low} GIC (Figure 4.13B). In addition, immunoblotting analysis also revealed that *GPR56* protein levels are lower in the presence of TNF- α (Figure 4.13C). Therefore, *GPR56* is downregulated during the PN to MES transition in response to TNF- α . Taken together, our results show that *GPR56* expression is inversely correlated with a mesenchymal phenotype in GIC, thereby suggesting that *GPR56* downregulation might be required for the MES transition in GBM.

4.2.4 Loss of *GPR56* promotes MES differentiation *in vitro* and *in vivo*

To test our hypothesis about the role of *GPR56* in the mesenchymal transition of GIC, we first tested whether *GPR56* downregulation is required for CD44 expression by loss-of-function studies. To this end, we used shRNA-mediated knockdown of *GPR56* in the CD44^{low} GIC. First, we characterized five different

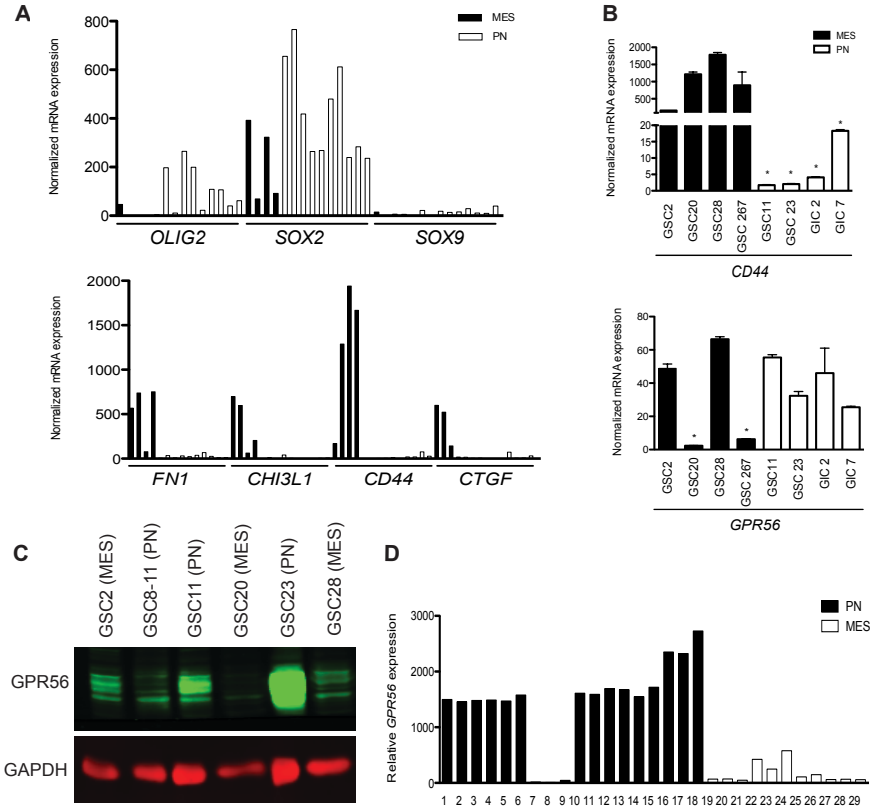


Figure 4.11: *GPR56* is enriched in PN-subtype GIC and inversely correlated with MES signature markers.

(A) RT-qPCR analysis of mRNA expression of *OLIG2*, *SOX2*, *SOX9*, *FN1*, *CHI3L1*, *CD44* and *CTGF* in MES (GSC2, GSC20, GSC28, GSC267) and PN (GSC8-11, GSC11, GSC23, GIC2, GIC4, GIC5, GIC6, GIC7, GIC8, GIC11) GIC. (B) *CD44* and *GPR56* mRNA expression was measured by RT-qPCR in three MES (GSC2, GSC20 and GSC28) and four PN (GSC11, GSC23, GIC2 and GIC7) GIC lines. Data are represented as mean $\pm$ SEM of at least three independent experiments. T-test was used to assess statistical significance. For *CD44*: * $p < 0.05$ in GSC267 vs GIC7, * $p < 0.005$ in GSC267 vs GIC2, * $p < 0.0005$ in the rest. For *GPR56*: * $p < 0.05$ in GSC267 vs GIC2, * $p < 0.005$ in GSC20 vs GSC23 and GIC2, * $p < 0.005$ in GSC20 and GSC267 vs GSC2, GSC11, GSC23, GSC28, GIC2 and GIC7. (C) Western blot analysis of GPR56 in different PN and MES GIC lines. GAPDH was used as loading control. (D) *GPR56* mRNA expression was assessed by microarray analysis in PN and MES GIC from the OSU (Ohio State University) dataset.

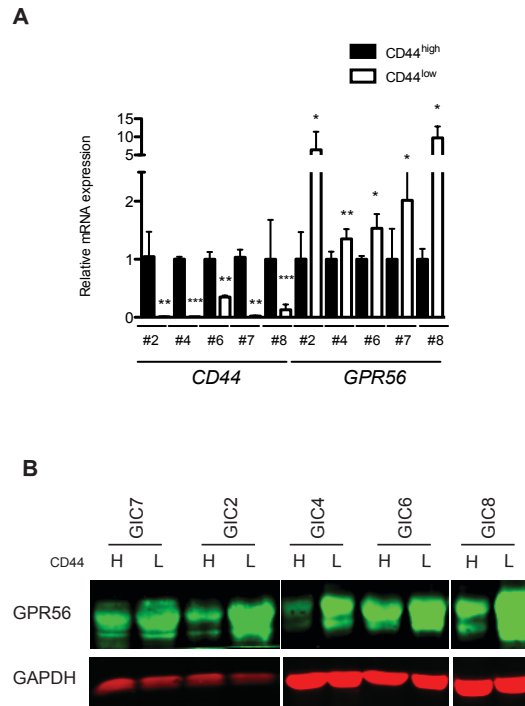


Figure 4.12: *GPR56* is highly expressed in $CD44^{\text{low}}$ GIC and downregulated in $CD44^{\text{high}}$ GIC.

(A) *CD44* and *GPR56* mRNA expression was measured by RT-qPCR in different GIC lines sorted into $CD44^{\text{high}}$ and $CD44^{\text{low}}$ subpopulations. Data represents mean $\pm$ SEM of at least 3 independent experiments (t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$). (B) Western blot analysis of *GPR56* in different GIC lines sorted into $CD44^{\text{high}}$ and $CD44^{\text{low}}$ subpopulations. GAPDH was used as loading control.

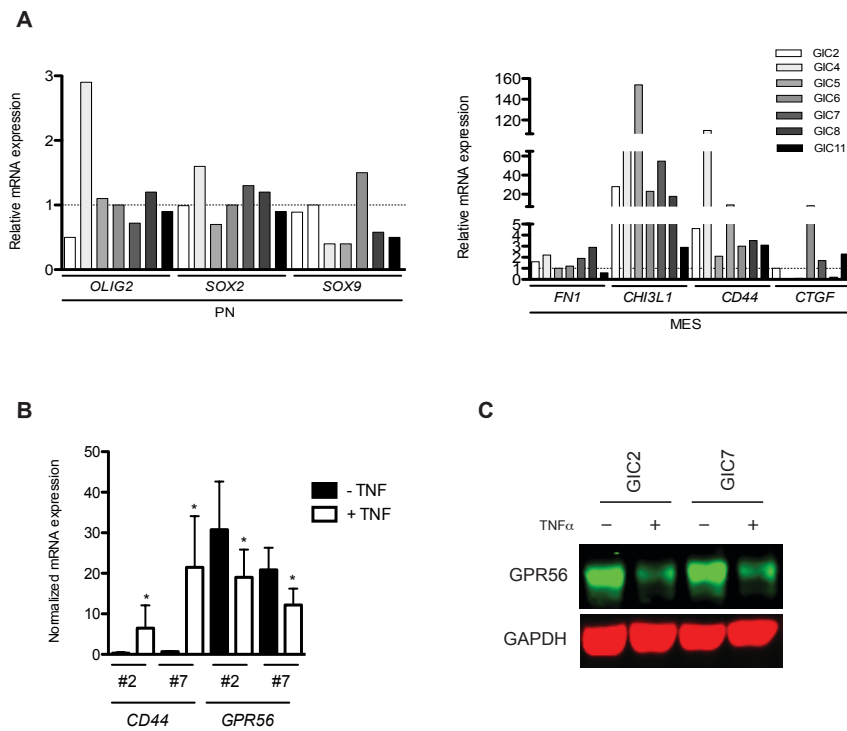


Figure 4.13: *GPR56* is downregulated during the MES transition of GIC in response to *TNF- α* .

(A) RT-qPCR analysis of mRNA expression of *OLIG2*, *SOX2*, *SOX9*, *FN1*, *CHI3L1*, *CD44* and *CTGF* in PN GIC (GIC2, GIC4, GIC5, GIC6, GIC7, GIC8, and GIC11) treated with or without *TNF- α* (10 ng/ml for 96h). (B) *GPR56* mRNA expression was measured by RT-qPCR in the *CD44*^{low} subpopulation of GIC2 and GIC7 treated with or without *TNF- α* (10 ng/ml for 96h). Data are represented as mean $\pm$ SEM of at least three independent experiments (t-test, * $p < 0.05$). (C) Western blot analysis of GPR56 in *CD44*^{low} GIC treated with or without *TNF- α* (10 ng/ml for 24h). GAPDH was used as loading control.

shRNA against *GPR56* by western blot analysis and RT-qPCR. *GPR56* shRNA #5 (SH5) was the hairpin with best knockdown efficiency and so we chose SH5 for our loss-of-function studies (Figure 4.14A, 4.14B and 4.14C). In line with our previous results, loss of *GPR56* in $CD44^{\text{low}}$ cells promoted an increase in *CD44* expression (Figure 4.14D and 4.14E). In addition, the increase in *CD44* expression in response to TNF- α was higher in *GPR56*-knockdown GIC (Figure 4.14D and 4.14E).

Since the perivascular space has been described as a niche for normal NSC and GIC alike (Calabrese et al., 2007; Charles et al., 2010), we wanted to study the receptor's function in an experimental setting that would require cell-to-cell contact, thereby better recreating the niche where GIC reside. To this end, we co-cultured GFP-tagged control- or *GPR56*-knockdown GIC with endothelial cells (EC) and determined the expression of the mesenchymal marker MCAM (melanoma cell adhesion molecule) by FACS analysis. In these more physiological conditions, *GPR56* loss also increased the expression of the MES marker MCAM (Figure 4.14F). As with other mesenchymal markers, the effect of *GPR56* knockdown on MCAM expression was enhanced in the presence of TNF- α (Figure 4.14F). To rule out the possibility that the flow cytometer was detecting doublets of GFP $^{+}$ GIC bound to MCAM $^{+}$ EC, we validated these results using ImageStream Imaging Flow Cytometry. As expected, GFP $^{+}$ /MCAM $^{+}$ cells detected by the flow cytometer were single GFP $^{+}$ cells co-expressing MCAM (Figure 4.14G).

Next, we asked whether loss of *GPR56* was able to induce a global MES transcriptional program. We selected a large and well-characterized MES gene signature (Bhat et al., 2013) and analyzed the mRNA expression of MES signature genes by RT-qPCR. Most MES genes were upregulated in *GPR56* knockdown-GIC (Figure 4.15A). Interestingly, the *GPR56* knockdown-induced increase in MES gene expression was larger than that induced by TNF- α treatment (Figure 4.15B). The induction of the MES signature upon *GPR56* loss was observed in the $CD44^{\text{low}}$ cell population but also in unsorted GIC (Figure 4.15C). Moreover, the increase in the MES signature was enhanced in *GPR56*-knockdown cells in the presence of TNF- α (Figure 4.15D). However, there wasn't a global reduction of the PN signature with *GPR56*-knockdown (Figure 4.16A). In line with our previous results, TNF- α treatment was also unable to decrease the expression of PN genes in $CD44^{\text{low}}$ GIC (Figure 4.16B). Noticeably, $CD44^{\text{low}}$ and $CD44^{\text{high}}$ GIC did not show significant differences in PN gene expression (Figure 4.16C).

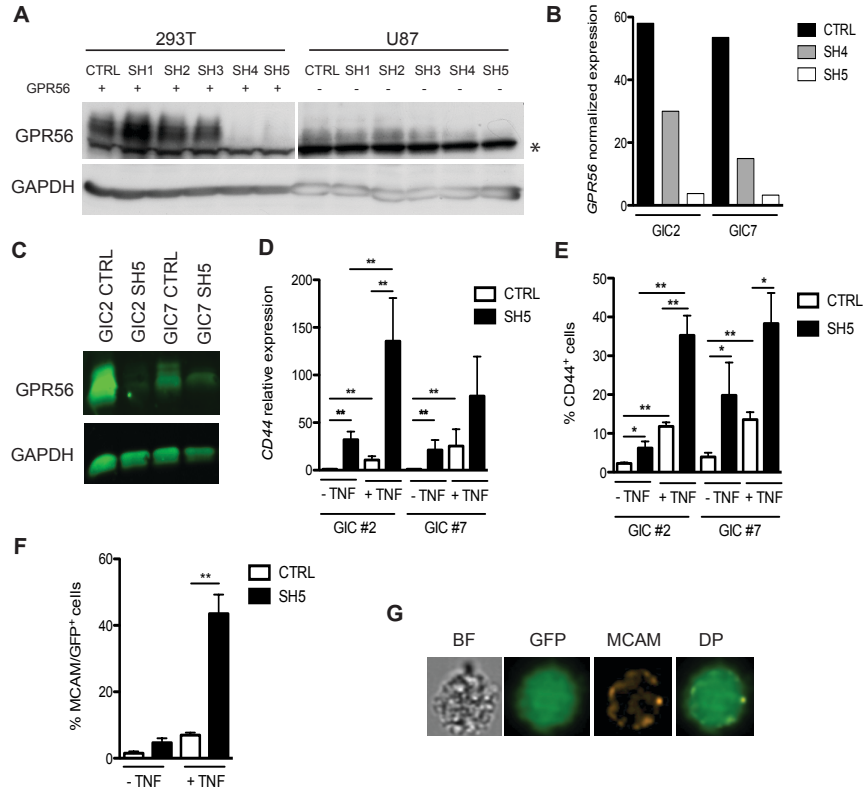


Figure 4.14: Loss of *GPR56* promotes the upregulation of the MES markers *CD44* and *MCAM*.

(A) Knockdown of overexpressed or endogenous *GPR56* was induced by transfection of five different shRNA against *GPR56* in 293T or U87 cells, respectively. *GPR56* protein levels were measured by western blot (*, non-specific band). GAPDH was used as loading control. (B) RT-qPCR analysis of *GPR56* mRNA expression in control- or *GPR56* knockdown-CD44^{low} GIC. (C) Western blot analysis of *GPR56* in CD44^{low} GIC2 and CD44^{low} GIC7 stably transduced with control shRNA (CTRL) or *GPR56* shRNA #5 (SH5). GAPDH was used as loading control. (D) *CD44* mRNA expression was measured by RT-qPCR in control- (CTRL) and *GPR56* knockdown (SH5)-CD44^{low} GIC in the presence or absence of TNF-α. Data are represented as mean ± SEM of at least three independent experiments (t-test, **p < 0.01). (E) FACS analysis of CD44⁺ cells in *GPR56* knockdown-CD44^{low} GIC treated with or without TNF-α for 4 days. Data are represented as mean ± SEM of at least three independent experiments (t-test, *p < 0.05, **p < 0.01). (F) FACS analysis of MCAM⁺ cells in GFP⁺/GPR56 knockdown- or GFP⁺/control-GIC co-cultured with GFP⁻ endothelial cells (EC). Data are represented as mean ± SEM of at least three independent experiments (t-test, **p < 0.01). (G) Representative images of GFP⁺/MCAM⁺ GIC co-cultured with GFP⁻ EC using ImageStream Imaging Flow Cytometry.

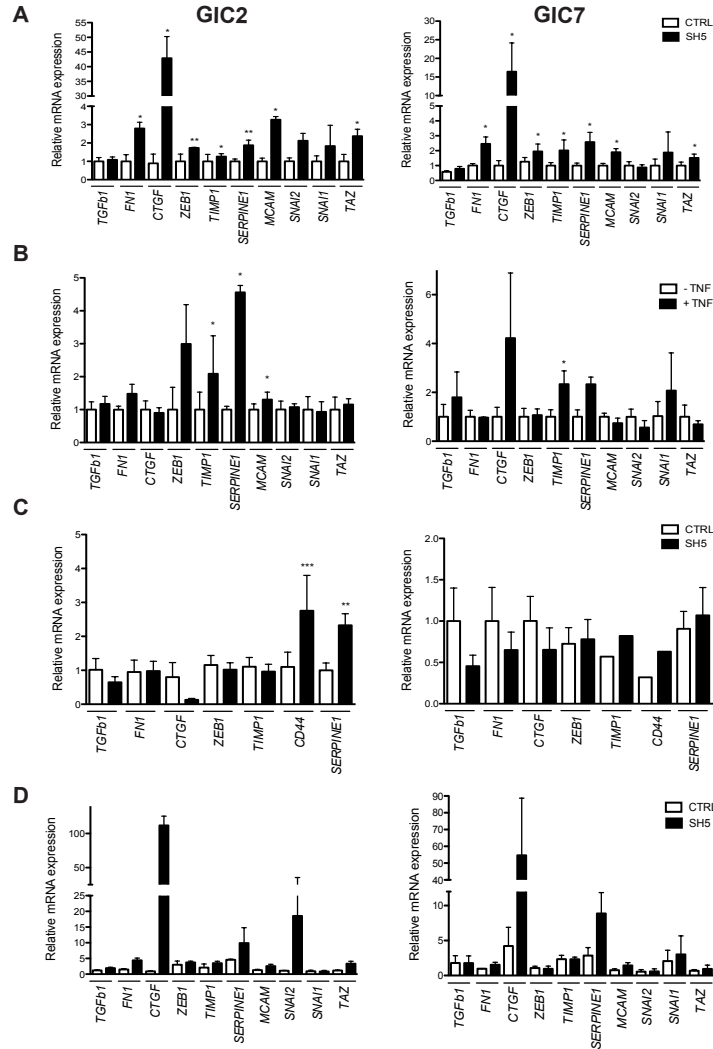


Figure 4.15: *Loss of GPR56 promotes a global MES differentiation.*

(A) MES signature in control- (CTRL) and *GPR56* knockdown (SH5)-CD44^{low} GIC was assessed by RT-qPCR of the following MES genes: *TGFB1* (transforming growth factor b1), *FN1*, *CTGF*, *ZEB1* (zinc finger E-box binding homeobox 1), *TIMP* (metallopeptidase inhibitor 1), *SERPINE1* (endothelial plasminogen activator inhibitor), *MCAM*, *SNAIL1*, *SNAIL2* (*snail family zinc finger 1 and 2*) and *TAZ* (tafazzin). (B) MES signature in CD44^{low} GIC treated with or without TNF- α (96h, 10 ng/ml) was assessed by RT-qPCR. (C) MES signature in whole population CTRL or SH5 was assessed by RT-qPCR. (D) MES signature in CTRL- and SH5-CD44^{low} GIC treated with TNF- α was assessed by RT-qPCR. Data are represented as mean $\pm$ SEM of at least three independent experiments (t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$).

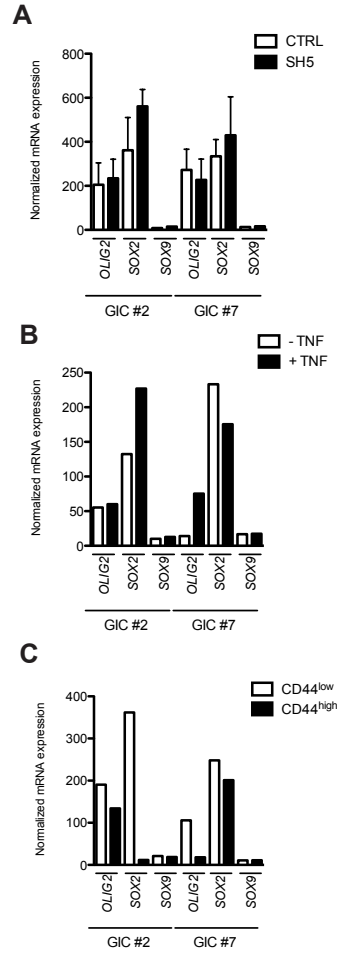


Figure 4.16: *Loss of GPR56 does not affect the PN signature.*

(A-C) PN signature of CD44^{low} GIC was assessed by RT-qPCR analysis of three PN genes *OLIG2*, *SOX2* and *SOX9*. (A) PN signature of control (CTRL) and *GPR56* knockdown-CD44^{low} GIC (SH5). Data are represented as mean $\pm$ SEM of at least three independent experiments (t-test). (B) PN signature of CD44^{low} GIC treated with or without TNF- α (96h, 10 ng/ml). (C) PN signature in CD44^{low} and CD44^{high} GIC.

In order to find a gene expression-based signature associated with GPR56 activity, we performed gene expression microarrays on paired *GPR56* knockdown-GIC (2 and 7) and control-GIC (2 and 7). A paired two-class Significance Analysis of Microarrays (False Discovery Rate < 5%) identified 117 genes differentially expressed between knockdown and control cells (53 up-regulated and 64 down-regulated) (Figure 4.17A and Table 4.2). As expected, *GPR56* gene was found in the list of genes significantly downregulated in the knockdown samples. Using these 117 genes, a GPR56 low activity-associated signature was derived and applied onto the TCGA gene expression dataset. The GPR56 low activity-associated signature was found highly enriched in MES-subtype GBM compared with the other GBM subtypes. Among the different GBM subtypes, the PN- (G-CIMP-) and G-CIMP⁺-GBM showed the lowest enrichment for this signature (Figure 4.17B).

In vivo, loss of *GPR56* also promoted the MES differentiation of tumors. Histologically, orthotopic tumors formed by intracranial injection of *GPR56* knockdown-GIC expressed higher levels of CD44 than control-GIC-induced tumors (Figure 4.18B). Interestingly, *GPR56* knockdown didn't affect overall tumor growth. There is a high degree of variability in tumor size between different animals, but differences were not significant when comparing tumors induced by *GPR56* knockdown-GIC *versus* control-GIC (Figure 4.18A). The latter result is consistent with our *in vitro* observations, whereby long-term cultured *GPR56* knockdown-GIC and control-GIC have the same proliferation rate (data not shown). However, at early stages after knockdown induction, loss of *GPR56* decreases GIC proliferation as measured by EdU incorporation (Figure 4.18C). This result may be explained by the fact that during the epithelial-to-mesenchymal transition (EMT), the cytoskeletal rearrangements necessary to lose cellular adhesion and acquire a higher motility phenotype are associated with a reduced proliferation rate (Evdokimova et al., 2009; Hugo et al., 2013). Indeed, the mesenchymal inducer TNF- α promotes a reduction in tumor size in mice orthotopically injected with proneural GIC, an effect that might be accounted for by a reduction in cell proliferation (Bhat et al., 2013). Accordingly, a recent study reported that *GPR56*-knockout mice have fewer mitotic neural progenitors than wild-type animals, suggesting that GPR56 promotes NSC proliferation (Bae et al., 2014). Therefore, the reduction in cell proliferation may be transitory during the initial steps of the EMT process. Overall, our findings indicate that loss of *GPR56* induces the MES differentiation of GIC both *in vitro* and in mouse orthotopic

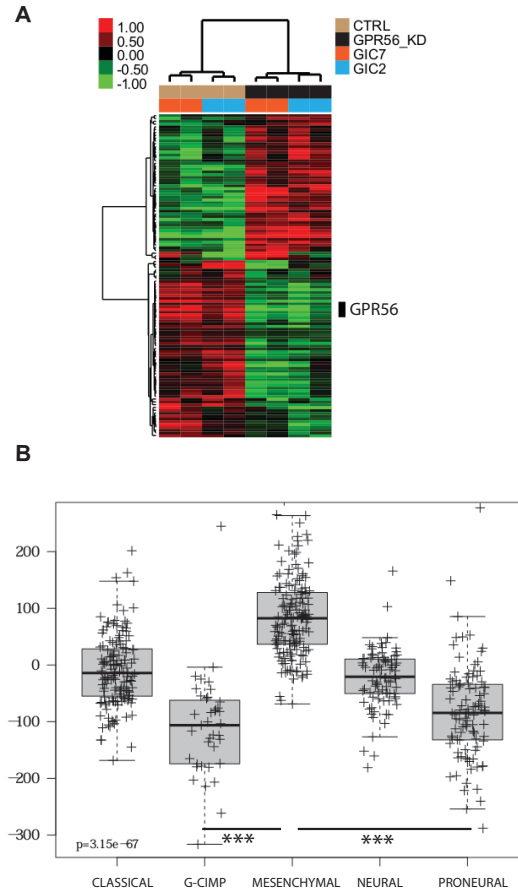


Figure 4.17: *GPR56* low activity-associated signature.

(A) A gene expression signature associated to low *GPR56* activity was derived from the microarray analysis of control- and *GPR56* knockdown-GIC (GIC 2 and GIC7). Shown is the heatmap of the genes differentially expressed between control- and *GPR56* knockdown-cells (paired two-class Significance Analysis of Microarrays, FDR < 0.05). (B) Expression of the *GPR56* low activity-associated signature across the different subtypes of GBM was retrieved from the TCGA dataset (Affymetrix U133A platform). Box-plot diagram showing the *GPR56* low activity-associated signature expression levels in patients with GBM (ANOVA, *** $p < 0.001$).

Results

A			B		
Gene ID	Fold Change	q-value(%)	Gene ID	Fold Change	q-value(%)
COX7C	1.65	0.00	GSTA4	0.60	0.00
C6orf145	2.05	2.99	LYPLAL1	0.57	0.00
MT1X	1.78	2.99	GPR56	0.15	0.00
SYPL1	1.53	2.99	FNDCA3	0.65	0.00
LHFP	2.82	2.99	CIDECP	0.62	0.00
ENST00000360485	2.60	2.99	CIDEC	0.65	0.00
MT1A	1.66	2.99	RTN1	0.25	0.00
ENST00000478163	2.56	2.99	DUS2L	0.55	0.00
C2CD2	1.84	2.99	LOC285141	0.28	0.00
RFPL4A	2.34	2.99	TMEM182	0.45	0.00
ST3GAL5	4.18	2.99	DCXR	0.42	0.00
APCDD1	1.56	2.99	C1orf106	0.68	0.00
SFRP2	2.76	2.99	OSCP1	0.36	0.00
STT3A	1.31	2.99	VPS4A	0.45	0.00
LIMK1	1.78	4.55	MBTPS2	0.72	0.00
MFAP4	6.49	4.55	B4GALNT4	0.64	0.00
PEX11B	1.66	4.55	PSAT1	0.52	2.99
C15orf44	1.40	4.55	EPDR1	0.64	2.99
RGS16	2.09	4.77	SCCPDH	0.71	2.99
SLC48A1	1.63	4.77	PTH2R	0.37	3.79
GLI3	1.82	4.77	C6orf118	0.61	3.79
PDZRN3	1.31	4.77	SLC7A1	0.58	3.79
CD200	2.15	4.77	FAM55C	0.52	3.79
D4S234E	7.19	4.77	OSBPL2	0.74	3.79
SLC2A4RG	1.73	4.77	SUMF1	0.73	3.79
TG	1.89	4.77	NCBP2	0.61	3.79
OTUD1	2.03	4.77	ATP6AP2	0.43	3.79
VSTM1	1.75	4.77	MECR	0.51	3.79
RHOA	1.95	4.77	CCT7	0.79	3.79
ODZ2	3.53	4.77	TRMT12	0.80	3.79
SMARCD2	2.61	4.77	FAM90A1	0.80	3.79
CABLES1	3.59	4.77	FAM116A	0.35	3.79
C14orf128	1.76	4.77	KIAA1279	0.59	3.79
MOB3C	1.48	4.77	SLC25A14	0.77	3.79
MT1L	2.09	4.77	KIAA0226L	0.71	3.79
TMEM145	1.66	4.77	DTL	0.53	3.79
CHRA1	1.90	4.77	GCC1	0.61	3.79
ANXA5	1.60	4.77	RNF216P1	0.71	3.79
ANLN	1.71	4.77	DNALI1	0.35	3.79
CAMK2N2	1.59	4.77	XLOC_014394	0.75	3.79
ARPC3	1.63	4.77	C2orf77	0.76	3.79
TULP3	2.32	4.77	PSMD2	0.47	3.79
EZR	2.07	4.77	TMX1	0.60	3.79
ANKRD9	1.44	4.77	LOC152217	0.75	3.79
EHD2	2.10	4.77	WVSD1	0.56	3.79
CARD10	2.84	4.93	EAF2	0.55	3.79
XLOC_000184	1.49	4.93	SPOPL	0.48	3.79
RGS3	1.50	4.93	VSIG10L	0.66	4.55
LASP1	1.30	4.93	ATP6V1B2	0.54	4.55
CYBRD1	1.75	4.93	MCART6	0.72	4.55

Table 4.2: *Genes differentially expressed in GPR56-knockdown versus control-GIC.*
(A-B) Microarray analysis of GPR56 knockdown-GIC. Table lists of the top 50 genes upregulated (A) and downregulated (B) in GPR56 knockdown-GIC.

xenograft models.

4.2.5 GPR56 mediates the adhesion of GIC to the extracellular matrix and endothelial cells

We next interrogated whether GPR56 would also regulate properties associated with a mesenchymal phenotype. Loss of cell-to-cell and/or cell-to-ECM contacts has been described as the first step in the acquisition of a MES phenotype. Accordingly, MES-subtype GBM have been associated with increased cell migration (Piao et al., 2013). GPR56 is an adhesion G-protein coupled receptor expressed in neural progenitors in the embryonic and adult brain playing a crucial role in cortical development (Piao et al., 2004). Knockout mice studies show that GPR56 mediates the attachment of radial glial endfeet to pial basement membrane (BM), thereby suggesting that GPR56 might regulate GIC adhesion to the ECM (Li et al., 2008; Luo et al., 2011). To explore this hypothesis, we performed cell adhesion assays on a matrigel matrix, which mimics the ECM. *GPR56* loss decreased cell polarization, with *GPR56*-knockdown GIC displaying a rounded-up morphology and creating clusters of aggregated cells as opposed to a monolayer of well spread cells with multiple contact points with the matrigel matrix in control-GIC (Figure 4.19A). This result suggests that GPR56 may be involved in the adhesion of GIC to the ECM.

Interestingly, some of the components of the pial BM are also present in the extracellular matrix surrounding blood vessels (BV). Since NSC and GIC reside in close apposition to BV (Calabrese et al., 2007; Charles et al., 2010), we asked whether GPR56 might mediate NSC/GIC localization to the perivascular niche. First, we performed immunohistochemical analysis of GPR56, SOX2 and CD31 in P28 adult mouse brains to determine the localization of GPR56 in neural progenitors in the subventricular zone (SVZ), where *GPR56* mRNA expression has been previously confirmed by fluorescence *in situ* hybridization (FISH) (Piao et al., 2004). As expected, GPR56 co-localized with SOX2 in the SVZ of the adult brain. It should be noted that SOX2 is expressed by both ependymal cells and cells in the subependymal layer where neural progenitors reside. GPR56 was expressed only in the subependymal layer. Therefore, GPR56 is expressed in SOX2⁺ neural progenitors in the adult mouse brain. In addition, GPR56 is expressed in cells within the SVZ that closely contact BV, suggesting that the receptor might mediate binding of adult normal neural progenitors to the

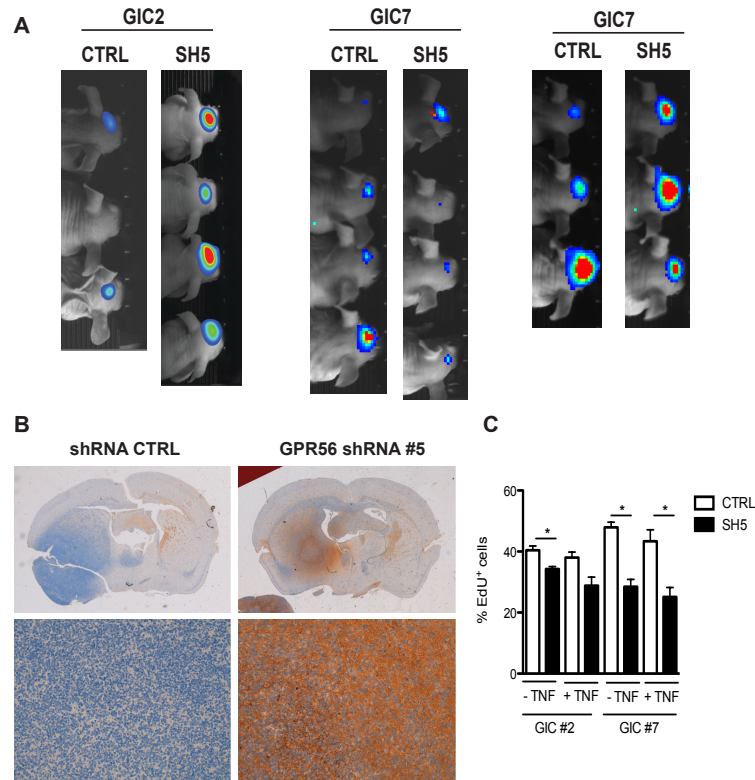


Figure 4.18: *Loss of GPR56 promotes MES differentiation in vivo.*

(A) Orthotopic tumors in immunocompromised nude mice were induced by intracranial injection of control- (CTRL) or *GPR56* knockdown (SH5)-GIC constitutively expressing firefly luciferase. Tumor volume *in situ* was assessed by bioluminescent imaging. (B) Immunohistochemistry of CD44 in CTRL-GIC2- or SH5-GIC2-induced orthotopic tumors. Representative stainings of 4 injected mice per group are shown. (C) Cell proliferation of CTRL-GIC and SH5-GIC was assessed by EdU incorporation. Data are represented as mean $\pm$ SEM of at least three independent experiments (t-test, * $p < 0.05$).

perivascular space (Figure 4.19B).

The above results suggest that GPR56 might also be mediating the adhesion of GIC to BV in analogy to their normal counterparts. To directly test this hypothesis, we performed adhesion assays of GFP-tagged GIC to a monolayer of brain microvascular endothelial cells (BMEC) treated with or without TNF- α . In this setting, *GPR56*-knockdown GIC displayed impaired adhesion to BMEC in the presence of TNF- α (Figure 4.19C), thus indicating that GPR56 mediates the adhesion of GIC to BMEC.

We have observed that loss of *GPR56* reduces GIC adhesion to EC and the ECM. To interrogate whether loss of cell adhesion would have an effect in tumor migration/infiltration *in situ*, we measured *corpus callosum* infiltration by magnetic resonance imaging (MRI) in human GBM (HCB dataset). GBM with low expression of GPR56 displayed a higher degree of *corpus callosum* infiltration than GPR56^{high} tumors, thereby suggesting that cells with low or no expression of GPR56 are more capable of migrating along the *corpus callosum*, one of the major routes of infiltration of human GBM cells (Figure 4.20A). Furthermore, the percentage of highly infiltrating tumors (> 50% of infiltrative tumor area) was higher in the group of GPR56^{low} GBM, while tumors with less than 20% of tumor infiltration expressed high levels of GPR56 (Figure 4.20B).

Taken together, our results point to GPR56 mediating the attachment of GIC to EC in the perivascular niche, while preserving their neural phenotype by preventing the acquisition of mesenchymal features. Since loss of adhesion is one of the hallmarks of EMT, we hypothesize that upon GPR56 inhibition GIC would lose adhesion to EC leading to the acquisition of a mesenchymal phenotype.

4.2.6 Loss of *GPR56* promotes radioresistance

Mesenchymal differentiation has also been linked to resistance to therapy. Several EMT-inducers such as ZEB1 or NF- κ B are associated with increased chemoradioresistance (Siebzehnrb et al., 2013; Bhat et al., 2013). Therefore, we asked whether GPR56 was involved in the acquisition of radioresistance. Since it has been described that ionizing radiation (IR) induces a shift in GBM-subtype signature from PN towards MES (Halliday et al., 2014), we first checked whether IR enhances MES differentiation in our GIC cultures by measuring MCAM expression. As expected, control-GIC increase MCAM expression in response to

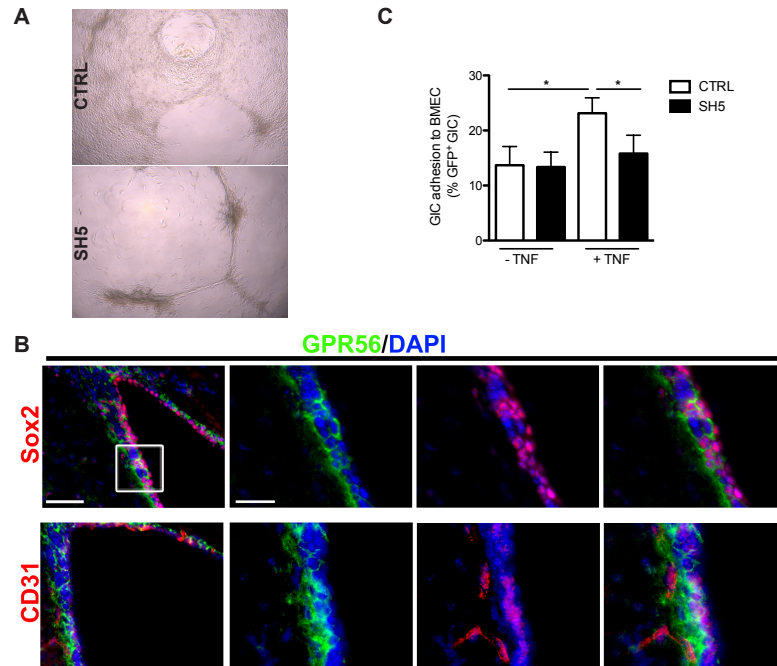


Figure 4.19: *GPR56* mediates the adhesion of GIC to endothelial cells preventing their MES transition.

(A) *GPR56* knockdown- and control-CD44^{low} GIC were cultured on top of a matrigel matrix (thick layer) in the presence of EC-conditioned medium for three days. Representative pictures of three independent experiments are shown. (B) Immunohistochemistry of *GPR56* (green), *SOX2* (red) and *CD31* (red) in P28 adult mouse brains. Scale bar: 50 μ m. Enlarged inset is shown in the right panels. Scale bar: 25 μ m. (C) Adhesion of GFP⁺ control- (CTRL) and *GPR56* knockdown (SH5)-GIC to a monolayer of brain microvascular endothelial cells (BMEC) was assessed by flow cytometry. BMEC had been previously treated with or without TNF- α (10 ng/ml) for 16h. Data are represented as mean $\pm$ SEM of at least three independent experiments (t-test, *p < 0.05).

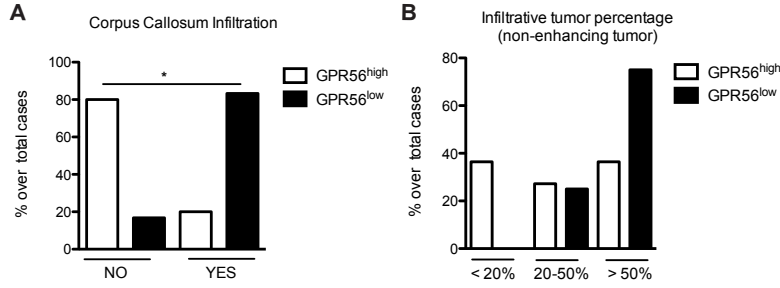


Figure 4.20: *Loss of GPR56 enhances tumor infiltration in human GBM.*

(A) Percentage of cases with or without *corpus callosum* infiltration in GPR56^{low}- or GPR56^{high}-GBM (Hospital Clínic de Barcelona dataset) (n = 21, Chi square test, *p < 0.05). (B) Percentage of cases with varying degrees of tumor infiltration in GPR56^{low}- or GPR56^{high}-GBM (Hospital Clínic de Barcelona dataset).

IR. In *GPR56*-knockdown GIC, the IR-induced increase in MCAM expression was higher than in control cells. Since MES-subtype GIC have been described to be more radioresistant (Bhat et al., 2013), we cannot rule out the possibility that the increase in MCAM expression is not merely due to an enrichment in the MES-subtype GIC subpopulation in our cultures because the PN-GIC have lower survival rates after exposure to IR. However, we observed similar cell death rates in control- and *GPR56* knockdown-GIC after IR (data not shown), so we concluded that *GPR56* knockdown enhances IR-induced mesenchymal differentiation (Figure 4.21A). Furthermore, we observed that IR promotes a reduction in *GPR56* expression, supporting the idea that *GPR56* downregulation is required for a PN to MES transition (Figure 4.21B).

Next, we examined whether loss of *GPR56* was sufficient to lead to radioresistance. In response to IR, PN-GIC usually undergo a profound arrest in G2/M. On the contrary, MES-GIC only display a modest arrest in G2/M upon exposure to IR, which correlates with a faster ability to repair DNA damage as measured by γ -H2AX foci formation (Bhat et al., 2013). According to the acquisition of a mesenchymal phenotype in *GPR56* knockdown-GIC, the number of cells in G2/M after IR was significantly lower in *GPR56* knockdown-GIC than in control-GIC (Figure 4.21C). TNF- α treatment reduces the number of GIC in G2/M in response to IR (Bhat et al., 2013). Strikingly, *GPR56* knockdown was more efficient than TNF- α at preventing G2/M arrest in CD44^{low} GIC. However, when using the unsorted total GIC population the reduction in

cells in G2/M was weaker in *GPR56* knockdown-GIC than in TNF- α -treated cells (Figure 4.21D). These findings suggest that GPR56 loss mainly affects the CD44^{low} cell population. We then measured the kinetics of γ -H2AX foci formation and repair in *GPR56* knockdown-GIC *versus* control-GIC. Although both cell lines showed comparable γ -H2AX foci formation at early time-points, *GPR56* knockdown-GIC showed enhanced repair ability 24h and 48h after IR (Figure 4.21E). Therefore, GPR56 inhibition promotes radioresistance of GIC.

4.2.7 Clinical association of GPR56

To test whether the effects of *GPR56* knockdown on the acquisition of a more radioresistant phenotype in cell culture could be translated to human GBM, we analyzed whether a GPR56 activity-associated signature correlates with overall survival (OS) or disease free survival (DFS) in GBM tumors of the TCGA dataset. Strikingly, GBM (G-CIMP⁺ and G-CIMP⁻) with a GPR56 low activity-associated signature displayed shorter OS (15.5 months *versus* 22.5 months) and DFS (6.5 months *versus* 9.1 months) than tumors with GPR56 high activity-associated signature (Figure 4.22A and 4.22B). To exclude the possibility that G-CIMP⁺ tumors included in the analysis were responsible for the improved survival phenotypes, we also analyzed this signature within G-CIMP⁻ only GBM. Importantly, the GPR56 low activity-associated signature also correlates with poor survival in G-CIMP⁻ GBM. The OS is 15.1 *versus* 20.1 months in GPR56 low activity and high activity GBM respectively, while the PFS is 6.2 months in GPR56 low activity and 8.4 months in GPR56 high activity GBM. Therefore, GBM patients with low GPR56 activity have worse prognosis, which might be explained by the increased radioresistance of GPR56^{low}-GIC in these tumors.

4.2.8 GPR56 prevents MES differentiation by inhibiting NF- κ B signaling

The NF- κ B signaling pathway is one of the main regulators of the MES transition in response to TNF- α (Edwards et al., 2011; Bhat et al., 2013). Since we observed that the TNF- α -induced MES differentiation is enhanced by *GPR56* knockdown, we interrogated whether GPR56 is interfering with NF- κ B signaling. In transcriptional assays using a luciferase reporter controlled by NF- κ B binding sites, *GPR56* knockdown-GIC displayed higher NF- κ B activity than

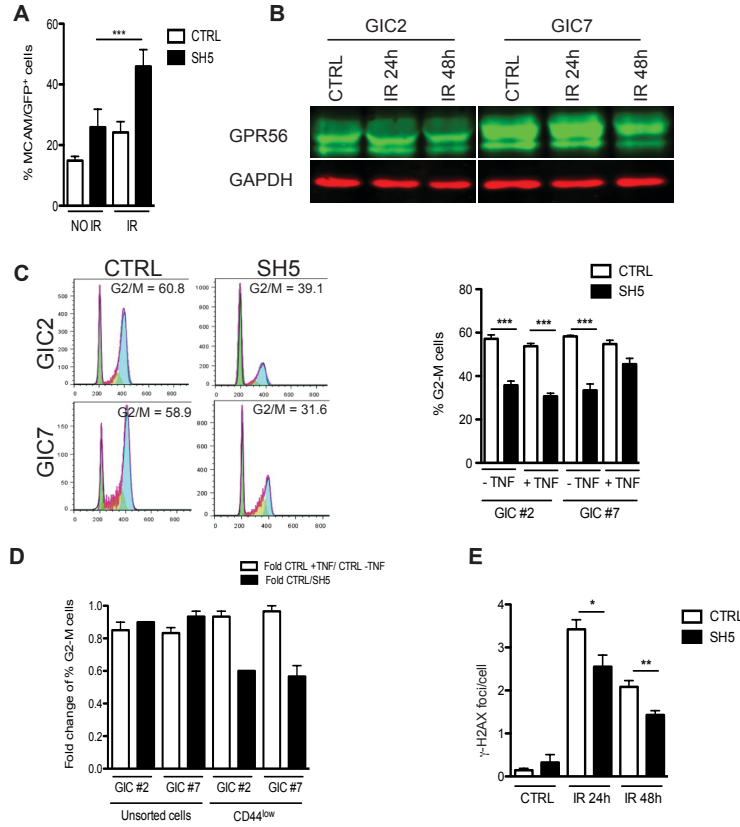


Figure 4.21: *Loss of GPR56 enhances tumor radioresistance in GIC.*

(A) FACS analysis of MCAM⁺ cells in GFP⁺/*GPR56* knockdown- or GFP⁺/control-GIC co-cultured with GFP⁺ endothelial cells for 8 days after exposure to 6 Gy of ionizing radiation (IR). Data are represented as mean $\pm$ SEM of at least three independent experiments (t-test, *** $p < 0.001$). (B) Western blot analysis of GPR56 in CD44^{low} GIC after 24h or 48h of treatment with 3 Gy of IR. GAPDH was used as loading control. (C) Cell cycle analysis of *GPR56* knockdown- and control-GIC. Left panel: representative cell cycle plots. The percentage of cells in G2/M phase is indicated within each plot. Right panel: percentage of cells in G2/M of GIC treated with or without TNF- α (96h, 10 ng/ml). Data are represented as mean $\pm$ SEM of at least three independent experiments (ANOVA, *** $p < 0.0001$). (D) Cell cycle analysis of *GPR56* knockdown- or control-GIC treated with or without TNF- α (96h, 10 ng/ml). Shown is the fold change of cells in G2/M in CTRL + TNF/CTRL - TNF (white bars) or CTRL/SH5 (black bars). Data are represented as mean $\pm$ SEM of at least three independent experiments (t-test, * $p < 0.05$). (E) γ -H2AX foci formation assay of *GPR56* knockdown- or control-GIC 24h or 48h after exposure to 3 Gy of IR. CTRL = non-irradiated cells. Data are represented as mean $\pm$ SEM of two independent experiments performed with 8 technical replicates (t-test, * $p < 0.05$, ** $p < 0.005$).

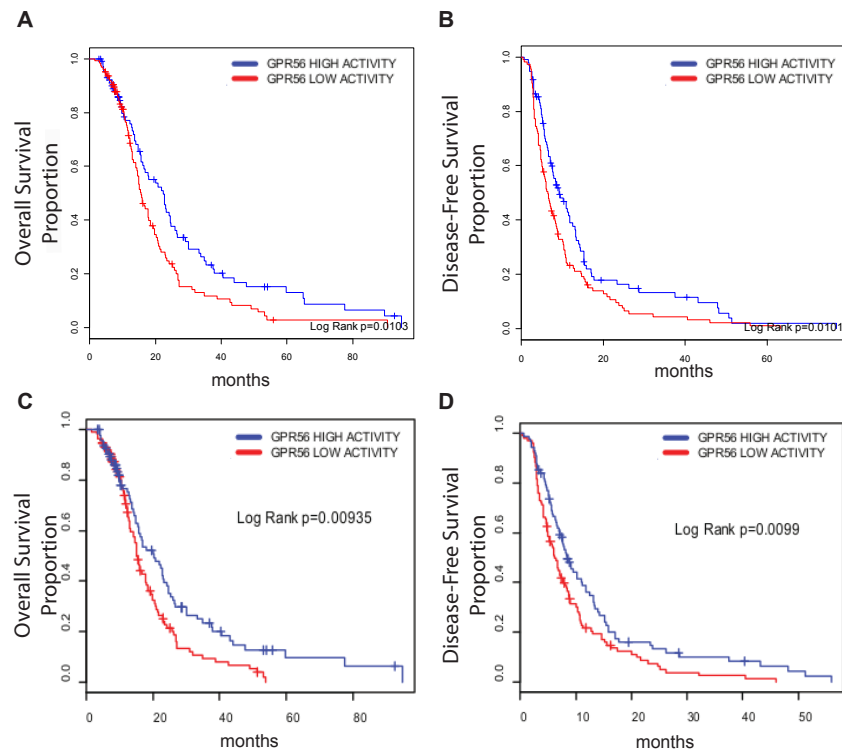


Figure 4.22: *GPR56* low activity-associated signature correlates with poor survival in human GBM.

(A-B) Kaplan Meier curves showing (A) overall survival (OS) and (B) disease-free survival (DFS) of GBM enriched in the *GPR56* low activity-associated signature *versus* GBM with low expression of this signature (TCGA dataset). (C-D) Kaplan Meier curves showing OS (C) and DFS (D) in G-CIMP⁺ only GBM enriched in the *GPR56* low activity-associated signature *versus* GBM with low expression of this signature (TCGA dataset).

control cells (Figure 4.23A). Next, we analyzed the localization of the NF- κ B member RelA/p65 in *GPR56* knockdown-GIC by immunocytochemistry (ICC). RelA/p65 protein was more concentrated in the nucleus of *GPR56* knockdown-GIC than in control-GIC (Figure 4.23B). Taken together, these results suggest that GPR56 inhibits NF- κ B-dependent transcriptional activity.

To further understand the mechanisms by which GPR56 inhibits NF- κ B activity, we analyzed the levels of I κ B α (nuclear factor kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), a canonical inhibitor of the NF- κ B signaling pathway (Gilmore, 2006). Interestingly, *GPR56*-knockdown GIC express lower levels of I κ B α than control-GIC (Figure 4.23C). In addition, while TNF- α decreased I κ B α levels in control-GIC as expected, the TNF- α -induced reduction of I κ B α was greatly enhanced in *GPR56*-knockdown GIC. These results are in agreement with the synergistic effects of TNF- α and *GPR56* knockdown observed on the mesenchymal phenotype. We next tested the effect of a constitutively active form of I κ B α in the context of *GPR56* knockdown (Figure 4.23D). I κ B α super-repressor (I κ B-SR) is a mutant that cannot be degraded by phosphorylation in response to NF- κ B agonists. Induction of CD44 by TNF- α was significantly inhibited by I κ B-SR both in control-GIC and *GPR56* knockdown-GIC (Figure 4.23E). These results suggest that GPR56 might inhibit NF- κ B signaling by maintaining the stability of I κ B α .

I κ B α protein levels are regulated by the IKK complex, which phosphorylates I κ B α and targets it for proteasomal degradation. IKK γ /NF- κ B essential modulator (NEMO) is the regulatory subunit of the IKK complex. To understand where in the pathway GPR56 is having its effect, we tested the effect of GPR56 loss in *NEMO*-knockdown GIC (Figure 4.23F). TNF- α wasn't able to induce CD44 expression in *NEMO*-knockdown GIC, as expected for an upstream activator of the NF- κ B signaling pathway (Figure 4.23G). Similarly, *GPR56/NEMO*-knockdown GIC had similar levels of CD44 as control cells both in the absence or presence of TNF- α , indicating that GPR56 inhibits NF- κ B signaling upstream of NEMO (Figure 4.23G and Figure 4.24B). Interestingly, while TNF- α does not significantly affect the levels of NEMO, *GPR56*-knockdown GIC display higher levels of NEMO protein (Figure 4.23H). Taken together, our results suggest that GPR56 inhibits NF- κ B signaling by reducing NEMO protein levels and thereby stabilizing I κ B α protein.

It has been suggested that activation of NF- κ B signaling by TNF- α precedes the induction of the STAT3 pathway (Bhat et al., 2013). Therefore, we tested

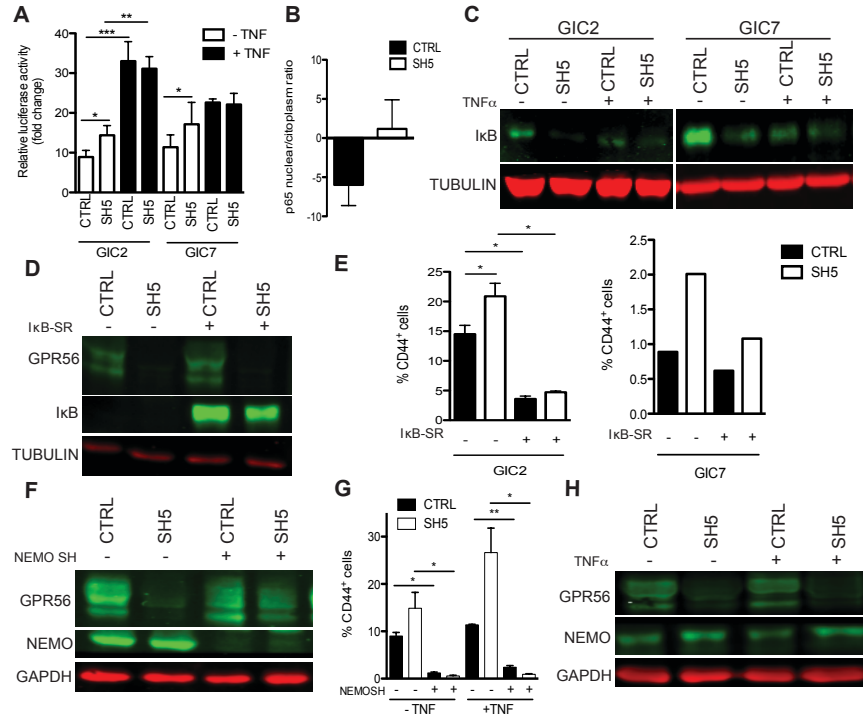


Figure 4.23: *GPR56* prevents *MES* differentiation by inhibiting *NF-κB* signaling.

(A) GIC were electroporated with a luciferase reporter gene controlled by *NF-κB*(RelA)-binding sites, together with a renilla expression plasmid to serve as an internal control, and treated with or without *TNF-α*. 48h later, cells were subjected to a dual luciferase assay. Data represents the mean $\pm$ SEM of at least three independent experiments (t test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$). (B) Quantification of the nuclear-to-cytoplasm ratio of p65 in control- or *GPR56* knockdown-GIC as assessed by immunocytochemistry. (C) WB analysis of IκBα in CD44^{low}/*GPR56* knockdown- and CD44^{low}/control-GIC treated with or without *TNF-α* (96h, 10 ng/ml). Tubulin was used as loading control. (D) WB analysis of GPR56 and IκBα in CD44^{low}/control- or CD44^{low}/*GPR56* knockdown-GIC infected with control- or IκB-SR-adenoviruses. Tubulin was used as loading control. (E) FACS analysis of CD44⁺ cells in control- or *GPR56* knockdown-GIC infected with control or IκB-SR adenoviruses. Cells were infected with the adenoviruses 24h prior to *TNF-α* treatment for an additional 72h. Data are represented as mean $\pm$ SEM of at least three independent experiments (t-test, * $p < 0.05$). (F) WB analysis of NEMO and GPR56 in CD44^{low}/control- or CD44^{low}/*GPR56* knockdown-GIC stably transduced with *NEMO* shRNA. GAPDH was used as loading control. (G) FACS analysis of CD44⁺ cells in control- or *GPR56* knockdown-GIC stably transduced with *NEMO* shRNA and treated with or without *TNF-α*. Data are represented as mean $\pm$ SEM of at least three independent experiments (t-test, * $p < 0.05$, ** $p < 0.01$). (H) WB analysis of NEMO and GPR56 in CD44^{low}/control- and CD44^{low}/*GPR56* knockdown-GIC treated with or without *TNF-α*. GAPDH was used as loading control.

whether a dominant-negative mutant of STAT3 (S3D) might also impair MES differentiation in *GPR56*-knockdown GIC (Figure 4.24A). As expected, S3D inhibited the TNF- α -induced upregulation of CD44 in control-GIC (Figure 4.24B). Surprisingly, we observed that S3D fails to suppress the induction of CD44 in *GPR56*-knockdown GIC (Figure 4.24B). Moreover, unlike to TNF- α treatment, *GPR56* knockdown fails to induce the phosphorylation of STAT3 (Figure 4.24C). These results indicate that loss of GPR56 rescues the inhibition of CD44 upregulation elicited by S3D, suggesting that GPR56 might act downstream of STAT3 activation. STAT3 is a transcription factor capable of either transactivating or repressing transcription of target genes (de la Iglesia et al., 2008). Therefore, STAT3 might be directly repressing *GPR56* mRNA expression. To test this idea, we analyzed *GPR56* mRNA levels in a previously reported microarray dataset of U87 glioblastoma cells that overexpress a constitutively active form of STAT3 (S3C) (Table 4.3 and Table 4.4) (de la Iglesia et al., 2008). *GPR56* mRNA levels were 2-fold lower in S3C-U87 than in control-U87 (Table 4.4). We also stably expressed S3C in GIC by retroviral infection and observed that S3C-GIC express lower levels of GPR56 protein than control cells (Figure 4.24D). Conversely, S3D failed to reduce GPR56 expression in response to TNF- α (Figure 4.24E). Taken together, these results suggest that STAT3 may be directly repressing GPR56 expression.

In summary, our results suggest that GPR56 inhibits NF- κ B signaling by stabilizing I κ B α protein levels via inhibition of the IKK complex, and that release of such inhibition to unfold a MES transition requires the presence of NEMO. Furthermore, STAT3 inhibits *GPR56* expression thereby contributing to enhance NF- κ B signaling. We propose a model in which TNF- α treatment activates STAT3, thereby promoting a decrease in *GPR56* expression and, consequently, NF- κ B pathway activation and MES differentiation.

4.2.9 Low GPR56 activity is associated to MES signatures across different tumor types beyond GBM

To interrogate whether GPR56 also inhibits mesenchymal differentiation in other tumor types, we analyzed our GPR56 low activity-associated signature across 25 different tumor types (TCGA datasets). Strikingly, we found a significant correlation between the GPR56 low activity-associated signature and signatures related to MES phenotypes in a great number of tumors (Figure

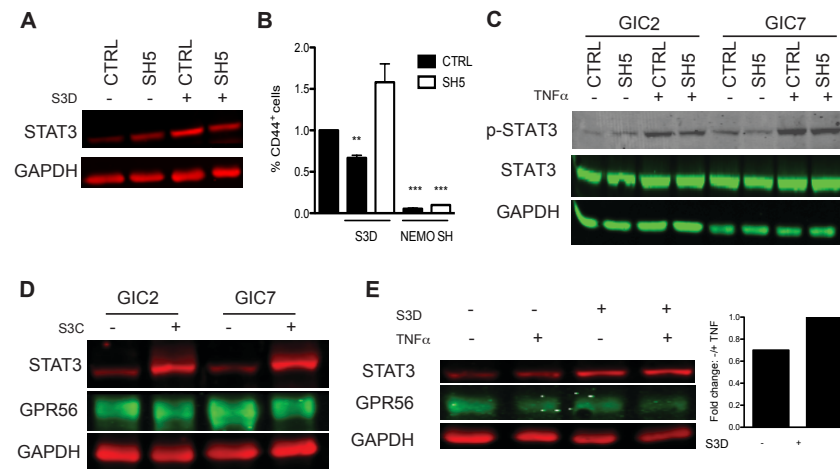


Figure 4.24: *GPR56* is downregulated by activated *STAT3*.

(A) Western blot (WB) analysis of STAT3 in CD44^{low} GIC stably transduced with S3D. GAPDH was used as loading control. (B) FACS analysis of CD44⁺ cells in control- or *GPR56* knockdown-GIC stably transduced with either S3D or *NEMO* shRNA. Data are represented as mean $\pm$ SEM of at least three independent experiments (t-test, **p < 0.01, ***p < 0.005). (C) WB analysis of total and phosphorylated STAT3 in CD44^{low}/control- or CD44^{low}/*GPR56* knockdown-GIC treated with or without TNF- α (96h, 10 ng/ml). GAPDH was used as loading control. (D) WB analysis of STAT3 and GPR56 in CD44^{low} control- or CD44^{low}/*GPR56* knockdown-GIC stably transduced with S3C. GAPDH was used as loading control. (E) Left panel: western blot analysis of STAT3 and GPR56 in CD44^{low} control- or CD44^{low}/*GPR56* knockdown-GIC stably transduced with S3D and treated with or without TNF- α . GAPDH was used as loading control. Right panel: quantification of GPR56 levels from the WB in left panel.

Genes Increased in S3C compared to Vector		
Name	Accession No.	Fold Increase
Hydroxysteroid (17-beta) dehydrogenase 2	NM_002153.1	4
Fibroblast growth factor 13	NM_004114.1	3
Hypothetical protein FLJ20399	NM_017803.1	2.9
Interleukin 13 receptor, alpha 2	NM_000640.1	2.6
Thrombospondin 3	L38969.1	2.4
Signal transducer and activator of transcription 3	BC000627.1	2.4
qz49h07.x1	AI493119	2.4
Tetraspan NET-6	NM_014399.1	2.4
Pleckstrin 2	NM_016445.1	2.4
Splicing factor, arginine/serine-rich 16	NM_007056.1	2.3
Alpha-ketoglutarate dehydrogenase complex	S72422	2.3
Homo sapiens cDNA DKFZp434G1835	AL117508.1	2.3
Homo sapiens chromosome 14 open reading frame 123	NM_014169.1	2.2
Forkhead box G1B	NM_005249.1	2.2
Ankyrin repeat and SOCS box-containing 6	BC001719.1	2.2
Nicotinamide nucleotide transhydrogenase	NM_012343.1	2.1
Neuronal protein (NP25)	NM_013259.1	2.1
Amyloid A4 protein	X06989.1	2.1
HLA class II region expressed gene KE2	NM_014260.1	2.1
Ribonuclease, RNase A family, 1 (pancreatic)	NM_002933	2.1
Homo sapiens clone 127 opioid growth factor receptor	AF172449.1	2.1
Polymerase (DNA directed), delta 1, catalytic subunit (125kD)	NM_002691	2.1
U2(RNU2) small nuclear RNA auxiliary factor 1	NM_006758	2.1
Homo sapiens C1orf24 mRNA	AF288391.1	2.1
SET and MYND domain containing 3	NM_022743	2.1
Homo sapiens cDNA oa80c10.s1	AA769006	2.1
Homo sapiens cDNA FLJ11118	AK001980.1	2
Actin, alpha 2, smooth muscle, aorta	NM_001613	2
Retinaldehyde-binding protein 1	NM_000326	2
HNOEL-iso protein	NM_020190	2
Brain and acute leukemia, cytoplasmic	NM_024812.1	2
DEAD (Asp-Glu-Ala-Asp) box polypeptide 10	NM_004398	2
MHC HLA-DQ beta mRNA	M32577	2
Calcium-regulated heat-stable protein 1 (24kD)	NM_014316	2
Hypothetical protein FLJ23311	NM_024680	2
Homo sapiens cDNA zr35c05.s1	AA195017	2
Transgelin	NM_003186	2
Kelch repeat and BTB (POZ) domain containing 4	NM_018095	2
SKB1 homolog (S. pombe)	NM_006109	2
Bromodomain adjacent to zinc finger domain, 1A	NM_013448	2
Matrix metalloproteinase 14 (membrane-inserted)	NM_004995	2
Homo sapiens cDNA yj89b06.s1	R72286	1.9
Transcription factor AP-4 (activating enhancer-binding protein 4)	NM_003223	1.9
Sorting nexin 6	NM_021249	1.9
Fas (TNFRSF6)-associated via death domain	NM_003824	1.9
Hypothetical protein FLJ10948	NM_018281	1.9
Nephropontin precursor	M83248	1.9
HT021	NM_020685	1.9
Hypothetical protein FLJ10970	NM_018286	1.9
Human DNA sequence from clone RP3-327J16 on chromosome 22q12.3-13.2	AL008583	1.9

Table 4.3: *Genes upregulated in S3C-expressing U87 glioblastoma cells.*

Microarray analysis of S3C-expressing U87 glioblastoma cells. Top 50 genes upregulated genes are shown.

Genes Decreased in S3C compared to Vector		
Name	Accession No.	Fold Decrease
Interleukin 8	NM_000584.1	5.1
Transglutaminase 2	BC003551.1	3.4
Mannose-specific lectin	U09716.1	3.3
Artemin gene	AF115765	3.2
Lethal giant larvae (Drosophila) homolog 1	NM_004140.1	3.1
Interleukin 8 C-terminal variant	AF043337.1	3.1
Hyaluronan synthase 1 (HAS1)	NM_001523.1	3
T-box 3 (ulnar mammary syndrome)	NM_016569.1	3
Complement component 3	NM_000064.1	3
Leukemia inhibitory factor	NM_002309.2	3
Protein tyrosine phosphatase, receptor type, F	NM_002840	2.8
Lectin, mannose-binding, 1	NM_005570	2.7
Thrombospondin 1	NM_003246	2.7
Lethal giant larvae (Drosophila) homolog 1	NM_004140	2.6
Tumor protein D52	NM_005079	2.6
Melanoma antigen, family C, 1	NM_005462.1	2.6
Chondroitin beta1,4 N-acetylgalactosaminyltransferase	NM_018371	2.6
Leukocyte-derived arginine aminopeptidase	NM_022350	2.5
Tissue inhibitor of metalloproteinase 3	NM_000362	2.5
Homo sapiens cDNA 7q53a12.x1	BF433429	2.5
ATP-binding cassette, sub-family C (CFTRMRP), member 3	NM_020037	2.5
Tissue inhibitor of metalloproteinase 3	NM_000362.3	2.5
DEAD-box protein p72	U59321.1	2.4
Metallothionein 1K	NM_176870	2.4
Protein inhibitor of activated STAT protein PIASx-alpha	AF077953.1	2.4
Homeo box A4 (HOXA4)	NM_002141	2.4
Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	NM_003654	2.4
Insulin receptor substrate-2 (IRS2)	AF073310	2.4
Tumor protein D52	NM_005079.1	2.4
Chromosome 21 open reading frame 97	NM_021941.1	2.3
Homo sapiens cDNA b24g09.x1	AI307760	2.3
Immunoglobulin superfamily, member 4	NM_014333	2.3
Tissue inhibitor of metalloproteinase-3	U67195	2.3
G protein-coupled receptor 56	NM_005682	2.3
Tumor protein D52	NM_005079.1	2.3
cDNA FLJ13511, highly similar to Homo sapiens 7h3 protein	AK023573.1	2.2
Hypothetical protein FLJ35036	NM_024724.1	2.2
NECDIN related protein	U35139	2.2
HERV-H LTR-associating 1	NM_005712.1	2.2
Cholesterol 25-hydroxylase	NM_003956	2.2
Homo sapiens cDNA wg58b07.x1, similar to protein phosphatase 2	AI760130	2.2
Zinc finger protein 175	NM_007147	2.2
Sequence from clone RP5-1054A22 on chromosome 20q11.22-12	AL031651	2.2
Matrix metalloproteinase 3	NM_002422	2.2
Tissue inhibitor of metalloproteinase 3	U67195	2.2
Transglutaminase	M98478	2.2
DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	NM_030881	2.2
Homo sapiens cDNA DKFZp566A0946	AL050069.1	2.2
Homo sapiens cDNA clone IMAGE:4593682	BG402105	2.1
Formyl peptide receptor 1	NM_002029	2.1

Table 4.4: Genes downregulated in S3C-expressing U87 glioblastoma cells.

Microarray analysis of S3C-expressing U87 glioblastoma cells. Top 50 genes downregulated genes are shown.

4.25A). Conversely, the GPR56 low activity-associated signature was negatively correlated with signatures related to epithelial differentiation (Prat et al., 2010; PMID: 20813035) and proliferation (Prater et al., 2014; PMID: 25173976) (Figure 4.25B). Interestingly, there was also a correlation between GPR56 low activity-associated signature and inflammatory signatures (Lim et al., 2009; PMID: 19648928), which correlates with the increased inflammatory infiltrates in MES-like tumors (Figure 4.25B). Therefore, GPR56 might have a general role as an inhibitor of the mesenchymal transition, presumably through the inhibition of NF- κ B signaling, across multiple tumor types.

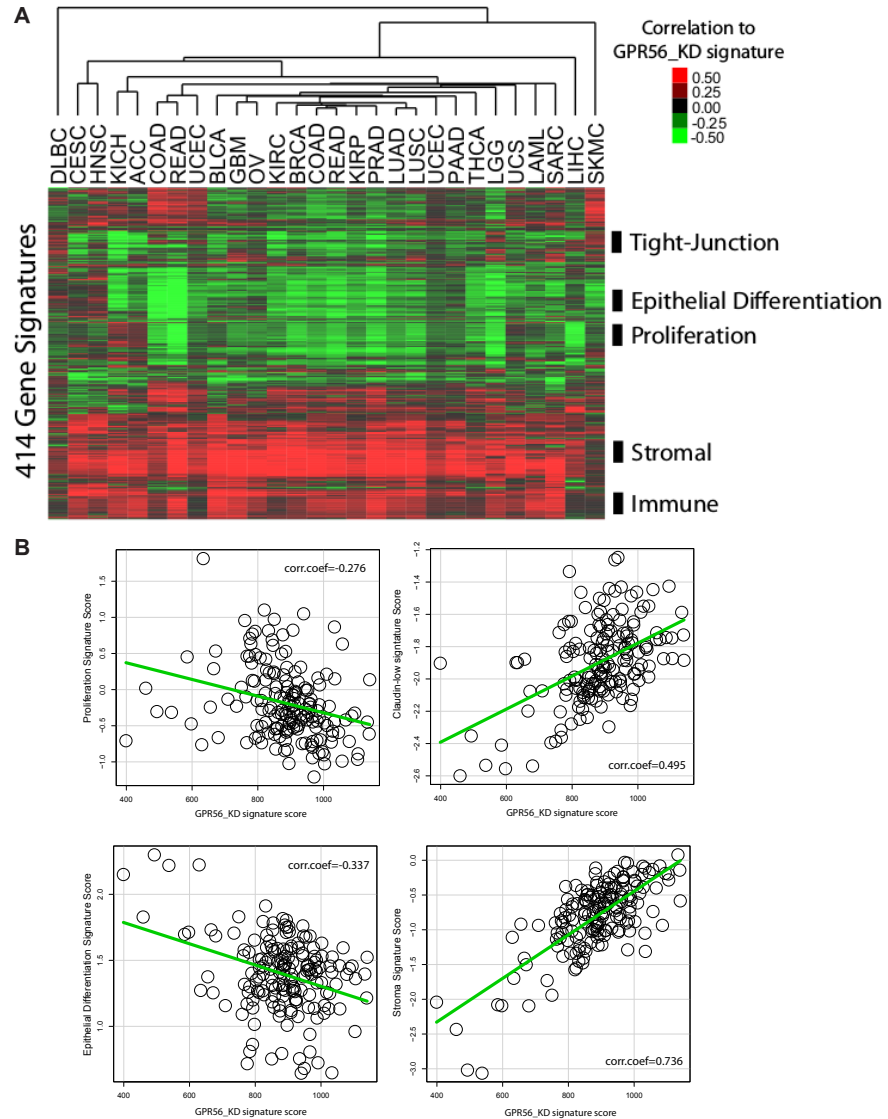


Figure 4.25: *GPR56* low activity-associated signature is associated to *MES* signatures across different tumor types.

(A) Correlation analysis between the *GPR56* low activity-associated signature and 414 gene signatures from the UNC (University of North Carolina) database across 25 different tumor types (TCGA datasets). (B) Individual correlation plots between the *GPR56* low activity-associated signature and selected gene signatures (proliferation, claudin-low, epithelial differentiation and stroma signature).

Discussion

GBM remains one of the most challenging tumors to understand and treat. The median survival time of GBM patients is only 12-15 months and despite huge efforts to improve the outcome of this deadly tumor, mostly all GBM patients succumb to the disease (Furnari et al., 2007). Although there have been recent advances in our understanding of the biology of GBM, those findings have not yet been translated into a significant improvement in patient survival. Therefore, there is an urgent need to better understand GBM biology and translating this knowledge into the clinic.

In GBM only a small fraction of cells within the tumor bulk that possess stem-like properties, known as glioma initiating cells (GIC), are considered to be the tumor driver cells (Piccirillo et al., 2009; Fomchenko and Holland, 2005). GIC and neural stem cells (NSC) share a huge number of similar characteristics, such as asymmetric cell division, self-renewal or pluripotency (Fomchenko and Holland, 2005). This cell population has the ability to initiate and propagate the tumor as well as to display resistance to conventional therapies. Therefore, understanding the biology of this cell population will help us to develop specific therapies targeting GIC and decrease the probability of relapse after treatment (Piccirillo et al., 2009). Indeed, previous knowledge of NSC regulation has been applied to the study of GIC biology for a better understanding of GBM behavior. Using this approach, several important pathways, such as Notch, Sonic-Hedgehog and Wnt, which are crucial in normal NSC biology, have been described as pivotal mediators during gliomagenesis (Visvader and Lindeman, 2008).

The main goal of this PhD thesis was to understand the mechanisms underlying GBM tumor formation and progression, with a special emphasis on the study of factors that have a role in normal NSC biology and might therefore be providing cancer cells with the stem-like properties required to acquire tumorigenic potential. To this end, this PhD thesis was divided into two separate projects. In the first project, the general aim was to get a deeper understanding of the factors that control NSC biology. We sought to identify novel factors involved in the maintenance of NSC under physiological hypoxic conditions. Using transcriptional profiling of hypoxic NSC, we have identified a novel TF that is active in NSC cultured under hypoxia and confers stem properties upon these cells. In the second project, using a candidate approach, we have identified a novel regulator of GBM progression from a proneural (PN) to a mesenchymal (MES) subtype. Accordingly, the discussion of this PhD thesis is structured into two different sections describing each of the above-mentioned projects.

5.1 Transcriptional profiling of hypoxic NSC identifies calcineurin-NFATc4 signaling as a major regulator of NSC biology

Most cells in multicellular organisms, including stem cells, live in an environment within tissues where oxygen levels are usually far below atmospheric oxygen concentrations. Physiologic oxygen levels may vary depending on cell type. In the brain, physiologic oxygen concentrations are at least one order of magnitude lower than in the atmosphere (from 2-5% in the cortex to 0.4% in pons) (Erecińska and Silver, 2001). Oxygen levels are known to affect cell transcription and, hence, their behavior. In the case of NSC, it has been described that physiologic hypoxia promotes cell population growth, survival, proliferation, and multicompetency (Studer et al., 2000; Chen et al., 2007; Pistollato et al., 2007). However, the molecular mechanisms by which low oxygen levels impact on normal NSC behavior are poorly understood. This is probably due to the fact that most functional studies of NSC in culture are done under non-physiologic 20% oxygen experimental conditions, thereby underestimating crucial factors that operate under physiologic hypoxic conditions.

Up until the beginning of this PhD thesis, only a candidate approach-driven

study had identified a couple of HIF targets that are, at least in part, implicated in mediating the NSC response to hypoxia (Studer et al., 2000). Therefore, there was an urgent need to understand the global impact of long-term physiologic hypoxia on the transcriptional status of NSC. In the first project of this PhD thesis, we have performed gene set enrichment analysis (GSEA) of microarray datasets from mouse NSC maintained in hypoxic *versus* normoxic conditions. GSEA is an analytical technique designed to test a priori defined gene sets for association with phenotypes. This approach was aimed at identifying pathways and transcription factors that are active in NSC cultured under oxygen concentrations that more closely resemble their normal tissue microenvironment. Using GSEA-TFT, we have shown that physiologically low oxygen levels activate a large number of TF, well beyond the HIF family of transcription factors. Furthermore, integration of TFT and pathway enrichment analysis has allowed us to uncover the calcium-regulated calcineurin-NFATc4-Id2 axis as a novel transcriptional program that controls NSC biology.

5.1.1 Physiologic hypoxia activates a large number of TF and has an impact on different signaling pathways

Culturing NSC at physiologic oxygen concentrations promotes an increase in proliferation and self-renewal (Studer et al., 2000; Chen et al., 2007). Therefore, we set out to identify the molecular mechanisms mediating the effect of low oxygen levels on NSC biology. The primary goal of the present study was to identify transcription factors that are active in physiologic hypoxic conditions. To this end, we performed GSEA using the TFT gene sets. This analysis revealed that physiologic hypoxia has a great impact on NSC transcriptional machinery: more than 110 TFT gene sets were enriched in hypoxic NSC, thereby suggesting that TF controlling expression of these genes might be activated in these conditions. As expected, HIF1 (hypoxia inducible factor 1) was one of these TF. We also identified other TF that had previously been described to be activated by hypoxia in other cell types, such as NF κ -B (RelA) or NFATc3 (Rius et al., 2008; de Frutos et al., 2007). Importantly, there are a large number of TFT gene sets that have a higher normalized enrichment score than HIF1 (HIF1 ranked in position #98 with a NES = 1.33 and FDR = 0.22), thus highlighting their potential role as important regulators of the hypoxic response. Strikingly, some of these of TF had never been reported to be activated by hypoxia. Therefore,

our results show that the hypoxic transcriptional response goes far beyond the sole activation of the HIF family of TF. We might speculate that the different TF that show up in our analysis are already engaged at the chromatin level as part of “dormant” transcriptional complexes, waiting for HIF transcription factors to be activated under low oxygen levels. Alternatively, the TF identified by our GSEA-TFT might be recruited independently of HIF to regulatory elements of their target genes in response to low oxygen levels. Further studies are needed to elucidate what molecular mechanisms are responsible for the broad transcriptional response of NSC to low oxygen levels.

Strikingly, E12 (E47/Tcf3) was the TFT gene set with the highest normalized enrichment score (NES), indicating this is the gene set that better correlates with hypoxia. E47/Tcf3 has been used to reprogram fibroblasts into NSC to obtain induced NSC (iNSC), thus suggesting that this TF has a key role in conferring neural stem properties upon NSC (Han et al., 2012). The fact that E12 is at the top of the list of the TFT gene sets that are enriched in hypoxia robustly validates our transcriptional profiling approach to identify factors that are important in NSC biology by searching for TFT enriched in hypoxic NSC.

In addition to the GSEA-TFT analysis, we performed a GSEA using the KEGG pathway gene sets. GSEA-KEGG pathway analysis of hypoxic NSC revealed an enrichment of genes within both the glycolytic and glycogen synthesis pathways which is consistent with a metabolic switch in response to hypoxia (Pescador et al., 2010; Iyer et al., 1998). During hypoxia, cells undergo an anaerobic switch that lowers mitochondrial oxidative phosphorylation activity, thereby reducing oxygen consumption. At the same time, there is an increase in glucose uptake and the rate of glycolysis (Papandreou et al., 2006; Aragonés et al., 2008; Kim et al., 2006). Consequently, the reduction of mitochondrial oxygen consumption limits the intracellular ROS production under hypoxic conditions (Tello et al., 2011). Therefore, the naturally hypoxic microenvironment of NSC might physiologically support a non-oxidative metabolism in these cells. In our experimental scenario, this hypoxic metabolic switch would be a physiological steady-state of the cell, and not so much a cell’s mechanism of defense in response to an environmental threat. Remarkably, several members of the aldehyde dehydrogenase family (*Aldh9a1*, *Aldh1a3*, *Aldh1b1*, *Aldh7a1* and *Aldh2*) significantly contribute to the enrichment signal in the glycolysis gene set. Interestingly, the ALDH family of enzymes have been described to be enriched in different types of stem and cancer stem-like cells (Armstrong et al., 2004; Corti et al., 2006; Bar

et al., 2007), supporting the notion that physiologic hypoxia might favor a stem cell phenotype. Taken together, these results suggest that the naturally hypoxic microenvironment of NSC might physiologically support a non-oxidative metabolism that promotes stemness.

Interestingly, the GSEA-KEGG pathway analysis also revealed enrichment in calcium signaling pathway members in NSC cultured in low oxygen concentrations. We observed an increase in the expression of calcium signaling mediators, including *Camk1g* (calcium/calmodulin-dependent protein kinase 1 gamma) and *Calml3* (calmodulin-like 3). At the same time, hypoxia promoted an increase in the expression of different calcium channels, including *Ryr1* (ryanodine receptor 1), *Cacna1c*, *Cacna1d* and *Cacana1h* (L-type voltage-dependent calcium channels, subunits alpha 1C, 1D and 1H, respectively). Ryanodine receptors are intracellular calcium channels that mediate calcium-induced calcium release (CICR) and have a major role in excitable cells such as neurons and muscle cells. Voltage-gated calcium channels are activated upon membrane depolarization. Interestingly, hypoxia induces membrane depolarization in some cell types, such as PC12 cells or carotid body cells (de Frutos et al., 2007; Zhu et al., 1996). This results in an increase in intracellular $[Ca^{2+}]$ by different mechanisms, including the activation of voltage-gated Ca^{2+} channels (Seta et al., 2004). Analogously, low oxygen levels might also induce membrane depolarization in NSC, thereby increasing intracellular $[Ca^{2+}]$. Both membrane depolarization and increased expression of calcium channels might contribute to a rise in the intracellular calcium concentration. On top of that, the increased levels of calcium signaling mediators might potentiate the cell's response to a given calcium concentration. All these events might have a profound effect on intracellular calcium signaling. Taken together, our results point to the calcium signaling pathway as a major regulator of NSC behavior under physiologic low oxygen levels.

On the other hand, pathways enriched in normoxic NSC included pathways indicative of a proliferative cell type, including basal TF, cell cycle and DNA replication. In agreement with this result, GSEA-TFT revealed that the gene targets of the TF E2F, one of the major regulators of the cell cycle (Müller and Helin, 2000), were the most significantly enriched in normoxic NSC. However, we have observed that NSC in hypoxic conditions have a higher rate of cell proliferation compared to normoxic cells. One simple explanation for these apparently paradoxical results is that pathways controlling proliferation in hypoxic

NSC may be fairly different from the ones regulating normoxic NSC proliferation. Alternatively, one might also propose that culturing NSC in normoxia might favor the expansion of a NSC pool that has adapted to proliferate under these conditions by upregulating and/or activating E2F. Therefore, culturing NSC under normoxic conditions might bias the culture to a NSC population that is not representative of the physiologic cellular state. In any case, these results highlight the importance of identifying the active signaling pathways that control proliferation under physiological hypoxia.

5.1.2 Role of calcium-induced calcineurin-NFATc4-Id2 signaling in NSC proliferation and self-renewal

Our GSEA study identified an increase in gene expression of members of the calcium signaling pathway in hypoxic NSC, and functional analyses using calcineurin inhibitors and siRNA-mediated *NFATc4* silencing suggest that the calcium-activated calcineurin-NFATc4 pathway promotes NSC proliferation and self-renewal both *in vitro* and *in vivo*. Calcium-regulated NFATs have a major role in vertebrate development and morphogenesis (Hogan et al., 2003; Wu et al., 2007; Li et al., 2011). These TF control early as well as late developmental processes ranging from early lineage specification in mouse embryonic stem cells to cardiac valve development (Li et al., 2011; de la Pompa et al., 1998; Ranger et al., 1998). In the nervous system, much is known about the role of NFAT in neurons. Calcineurin-NFAT signaling regulates neuronal survival and maturation, including dendritic/axonal growth and synaptogenesis (Ding et al., 2013; Schwartz et al., 2009; Benedito et al., 2005; Graef et al., 1999). Furthermore, knockout studies have shown that calcineurin-NFAT signaling is essential for neuregulin-mediated Schwann cell differentiation (Kao et al., 2009). However, the role of calcineurin-NFAT signaling in the biology of NSC was poorly understood.

In this study, we report for the first time that NFATc4 promotes self-renewal and proliferation of mouse NSC. In agreement with our results, there are evidences in the literature suggesting that calcium signaling might promote NSC properties. Specifically, purinergic signaling, which activates P2Y receptors and induces intracellular calcium transients, promotes mouse NSC expansion (Lin et al., 2007). In this context, we speculate that P2Y-induced calcium transients might be activating calcineurin-NFATc4, thereby promoting NSC proliferation.

Another study had reported that suppression of Wnt signaling by calcineurin-NFAT induces NSC to exit the cell cycle and promotes their differentiation in the chick embryo (Huang et al., 2011). Although these results apparently point to an opposed role for NFATs in NSC biology, this study shows that NFATc1 is the NFAT family member which inhibits Wnt signaling in the chick embryo, thereby inhibiting NSC proliferation. Taken together, these results suggest that NFATc1 and NFATc4 play opposing roles in NSC biology, although NFAT factors often play redundant functions. Interestingly, we have shown that NFATc1 is poorly expressed and it is mainly localized in the cytoplasm in mouse E13 NSC, while NFATc4 is highly expressed and heavily concentrated in the nucleus. Therefore, this might be a mechanism to counteract NFATc1 pro-differentiating functions in hypoxic NSC.

Since some NFATc4 targets are upregulated by hypoxia in a NFATc4-dependent manner, we hypothesize that NFATc4 is promoting self-renewal through the upregulation of one or more of these factors. Inhibitor of differentiation/DNA binding 2 (ID2) is one of these NFATc4 target genes. ID2 is a basic helix-loop-helix transcription factor that is crucial in cortical development because it preserves the neural stem cell state and prevents neuronal differentiation by antagonizing proneural and OLIG basic helix-loop-helix transcription factors (Wang et al., 2001; Paoletta et al., 2011; Ross et al., 2003). However, ID2 is still present in differentiated cells where it is required for axon growth arrest of post-mitotic neurons (Lasorella et al., 2006). Importantly, ID2 levels are crucial to determine its function: ID2 levels are very high in stem cells but it is downregulated in neurons where it no longer contributes to self-renewal and proliferation (Iavarone and Lasorella, 2006). Of note, *Id2* levels were not significantly downregulated in NFATc4 siRNA #1, which correlates with an inability of siRNA #1 to inhibit self-renewal. In this scenario, hypoxia-activated NFATc4 might promote *Id2* gene expression to ensure that *Id2* levels are high enough to keep NSC stemness and prevent differentiation. This might also provide a plausible explanation to the fact that NSC cultured in normoxia have an increased tendency to spontaneously differentiate. Taken together, our results suggest that NFATc4 might control NSC proliferation and self-renewal by promoting the expression of TF, including *Id2*, that contribute to the maintenance of the neural stem cell phenotype.

5.1.3 NFAT signaling and cancer

The cancer stem cell model proposes a clonally derived brain tumor arising from a cancer cell with stem-like properties. This cancer stem cell shares characteristics with normal stem cells, thereby suggesting that understanding normal stem cell regulation would help us better understand cancer cell biology (Fomchenko and Holland, 2005). In this study, we have identified the calcium signaling pathway as an important regulator of NSC behavior under physiologic low oxygen concentrations. Analogously, calcium signaling might be playing an important role in the biology of glioma stem-like cells (GIC). Indeed, remodeling of calcium homeostasis is an important event in promoting cancer malignant behaviors. Calcium signaling controls key aspects of tumorigenesis, such as cell proliferation, invasive migration, cell death, necrosis and autophagy (Chen et al., 2013). However, the role of this pathway in the behavior of GIC is still poorly understood and future studies are needed to unravel how calcium signaling might be regulating gliomagenesis.

In this PhD thesis, we have identified calcium-induced calcineurin-NFATc4 signaling as one of the major regulators of NSC biology. Therefore, studying the role of NFATc4 in the biology of GIC would represent a new approach to the study of gliomagenesis. Several recent findings have pointed to important roles for NFAT in modulating phenotypes associated with malignancy and tumor progression, such as migration, invasion and angiogenesis (Mancini and Toker, 2009). NFAT proteins often play redundant functions, however isoform-specific differences exist between NFATc1-4 during tumorigenesis. Direct and opposing roles for NFATc1 and NFATc2 in tumor development have been identified whereby NFATc2 functions as a tumor suppressor while NFATc1 behaves as an oncogene (Robbs et al., 2008). Additionally, NFAT have been suggested to participate in versatile functions during tumor progression. As an example, NFATc4 displays tumor suppressor activities during cell transformation (Li et al., 2006), while it enhances estrogen receptor transcriptional activity, thereby promoting cancer growth in breast cancer cells (Zhang et al., 2005). In GBM, NFATc4 behaves as an anti-tumorigenic factor by promoting doxorubicin-induced cell death (Gopinath et al., 2009). These apparently paradoxical results highlight the pleiotropic functions of NFAT proteins in tumorigenesis, which are mirrored by their pleiotropic nature during development. In this study, we have uncovered how oxygen levels regulate the calcium-responsive calcineurin-NFATc4-Id2 axis, thereby identifying a novel transcriptional program that con-

trols NSC biology. Our results highlight the importance of the microenvironment in the mechanisms of NFAT regulation. Therefore, understanding the role of NFAT in a physiological microenvironment is crucial for a deeper understanding of its role during tumorigenesis. Given the poor knowledge of NFATc4 function in GBM biology, the identification of the pivotal role of calcineurin-NFATc4-Id2 axis in NSC biology might also have important consequences in the glioma field.

5.1.4 Concluding remarks

Overall, we have undertaken a hypothesis-generating genomic approach to be used by the scientific community in future studies to investigate the role of our GSEA-identified TF in NSC biology. Therefore, our study will help get a deeper knowledge of the mechanisms by which NSC self-renew, proliferate and differentiate. In addition, the identification of hypoxia-driven NSC factors will help us characterize novel key factors that, besides playing a role in normal NSC biology, might also be involved in the maintenance and differentiation of glioma stem-like cells.

Furthermore, a deeper knowledge of the transcriptional machinery that controls NSC functions will help us improve the generation of induced NSC (iNSC). Therefore, our results might ultimately impact the way we generate iNSC to be used for autologous cell transplantation to treat CNS disorders by cell replacement therapies. Finally, our results may also have an impact beyond the NSC field, since other stem cell types, including embryonic and somatic stem cells, also reside in hypoxic microenvironments.

5.2 GPR56 is a NSC factor that restricts the proneural to mesenchymal transition in GBM by inhibiting the NF- κ B pathway

The molecular classification of human GBM into different subtypes has shed light into the biological processes that might be driving GBM pathogenesis and has been a major step forward into our understanding of this aggressive disease (Verhaak et al., 2010; Phillips et al., 2006). Every molecular subtype holds different types of oncogenic mutations affecting different major signaling pathways, which may suggest that every subtype is a different entity and that subtype-specific therapies targeting specific pathways should lead to better clinical outcomes. However, there is an inherent plasticity of glioblastoma cells to transition from one subtype to another and this shift in GBM subtype is often observed at recurrence (Phillips et al., 2006; Bhat et al., 2013). Specifically, a mesenchymal shift is often observed in PN-subtype tumors after ionizing radiation (IR) (Phillips et al., 2006; Halliday et al., 2014; Ozawa et al., 2014). Bioinformatic analyses coupled to mouse modeling studies have recently suggested that even primary GBM (G-CIMP⁻) don't arise de novo through independent driver mutations, but probably evolve from a common proneural-like precursor glioma (Ozawa et al., 2014). Therefore, the different GBM subtypes might represent different stages in tumor evolution, with the MES subtype being one of the most advanced stages. Identification of the molecular mechanisms that control transitions from one subtype to another will help us understand GBM tumor evolution. We believe that identifying molecular drivers of MES transition is a crucial step in the development of patient-tailored therapies in GBM.

In this study, we have described for the first time that an adhesion GPCR controls the transition between different cellular states. We show that inhibition of GPR56 promotes mesenchymal differentiation and radioresistance of GIC and its expression correlates with longer survival. Furthermore, we have identified GPR56 as a novel inhibitor of the NF- κ B signaling pathway, providing a mechanism by which this receptor prevents the MES transition.

5.2.1 GPR56: stem cells and tumors

The gene expression profile of GBM subtypes resembles that of different cell types or tissues, such as brain tissue and cells within the neural lineage including NSC in the PN subtype, or bone, smooth muscle and endothelial cells in the MES subtype (Verhaak et al., 2010; Phillips et al., 2006). This might be explained by all GBM subtypes having a common cell of origin with neural stem-like properties, and that the phenotypes observed in the different GBM subtypes are a consequence of the differentiation or transdifferentiation of this neural stem-like cell along different paths (Galli et al., 2004; Singh et al., 2004). Indeed, normal NSC have been described to be able to transdifferentiate into cells outside the neural lineage, such as endothelial cells and muscle cells (Galli et al., 2000; Wurmser et al., 2004). In analogy with their normal NSC counterparts, tumorigenic neural stem-like cells might also undergo transdifferentiation into these cell lineages thereby contributing to different tumor phenotypes (Chen et al., 2013; Ricci-Vitiani et al., 2010; Wang et al., 2010; Soda et al., 2011).

Alternatively, we propose that each GBM subtype is driven by different glioma-initiating cells at different stem-like stages with distinct differentiation potential. Each of these GIC would give rise to a tumor-bulk progeny according to their differentiation capabilities that would eventually contribute to shape the four different GBM tumor phenotypes. We hypothesize that in PN-subtype GBM, the glioma stem-like initiating cell has NSC properties. However, during GBM tumor evolution towards a MES subtype, the glioma stem-like initiating cell may progressively lose neural commitment while acquiring a less restricted program, eventually displaying characteristics of pluripotent embryonic stem cells (ESC). Indeed, an embryonic stem cell-like gene expression signature has been associated with poorly differentiated human tumors, including GBM (Ben-Porath et al., 2008). The fact that NSC can be easily reprogrammed into iPS by the sole expression of *OCT-4* supports this idea and suggests that the differentiation potentials of NSC and ESC might be highly related (Kim et al., 2009). Therefore, we believe that a deeper understanding of cell fate-restricting factors can shed light into GBM progression and adaptation. A better comprehension of the spectrum and dynamics of cellular states in GBM emerges crucial for establishing faithful models and developing therapeutic strategies.

In this PhD thesis, we report how GPR56, a NSC-enriched factor, is negligibly expressed in MSC and ESC and progressively upregulated during neural

lineage commitment. Similarly, *GPR56* is upregulated during the differentiation of human fetal muscle cells (Wu et al., 2013). Specification of cellular fate during development is a dynamic process by which diverse phenotypes are established in precise temporal and positional patterns. ESC follow a hierarchy of lineage-specific paths towards progenitors and tissue-specific cells accompanied by limited lineage potential (Strauss et al., 2012). In this context, *GPR56* might be behaving as a lineage specification factor that restricts ESC potential. Analogously, *GPR56* is enriched in PN-subtype GBM and lost during GBM tumor evolution towards a MES subtype. We hypothesize that *GPR56* might restrict a neural cell fate during development, while in cancer, *GPR56* inhibition might promote loss of a PN phenotype and acquisition of ESC-like properties. We suggest that the PN subtype, which has been linked to proneural developmental genes, might resemble a more restricted cell fate stage similar to NSC. Conversely, during the PN to MES transition, cancer cells might acquire a stem-like phenotype similar to earlier developmental stages (ESC-like stage) with higher plasticity, which might promote less differentiated tumor phenotypes with higher rates of transdifferentiation (Figure 5.1).

Here, we have identified a NSC-enriched factor progressively up-regulated during the neural-lineage specification of ESC as a pivotal player in the PN to MES transition in GBM. Through the study of its differential pattern of expression between NSC and ESC, we have identified an important player in GBM progression. However, further studies facing the role of *GPR56* in normal NSC are required. Additionally, low levels of *GPR56* are also associated to the acquisition of mesenchymal features in other tumor types, whereas high expression of *GPR56* is related to epithelial-like phenotypes. It is plausible to think that loss of *GPR56* in other types of stem-like cells might also lead to the acquisition of a gene expression program characteristic of a less restricted cell type such as pluripotent ESC. Therefore, these results strengthen the idea that understanding the mechanisms that promote the reacquisition of a less committed stem-like stage might help us to understand tumor progression. Finding out what are the factors that promote/inhibit stem cell specification during development might help identify the players that promote/inhibit plasticity during tumorigenesis.

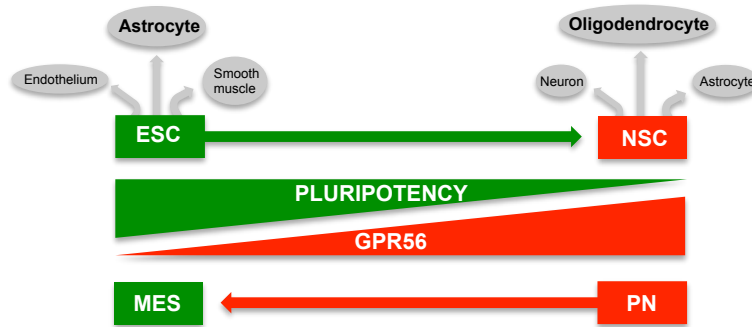


Figure 5.1: *Model depicting parallels between GBM tumor subtypes and stem cell lineages.*

Pluripotent ESC express low levels of *GPR56*, while more-committed multipotent NSC express high *GPR56* levels. Analogously to NSC, PN-subtype GBM express high levels of *GPR56* and oligodendrocytic markers. Therefore, PN-subtype GBM are NSC-like tumors. Conversely, *GPR56*-low MES-subtype tumors are characterized by the expression of non-neural markers which brings them phenotypically closer to ESC.

5.2.2 GPR56: phenotype and signaling

Besides *GPR56* being enriched in PN-subtype and downregulated in MES-subtype GBM, we further show that *GPR56* has a functional role as an inhibitor of MES differentiation. *GPR56* knockdown in GIC activates a global MES transcriptional program that includes a large number of mesenchymal drivers. Furthermore, loss of *GPR56* promotes the acquisition of properties associated with a mesenchymal phenotype such as radioresistance and loss of cell adhesion. Importantly, at the clinical level a low *GPR56* activity gene signature is associated with a poorer prognosis in human GBM patients.

In this study, we have described that the *GPR56* low activity-associated signature is highly enriched in MES-subtype GBM. Most importantly, we have found that a poorer patient prognosis is associated with low *GPR56* activity in GBM patients. Our results provide new insights into the molecular classification of GBM and point to the *GPR56* low activity-associated signature as a new tool to better classify GBM. The molecular classification of tumors is of crucial interest for clinical diagnostic, as well as to predict response to treatments and eventually develop patient-tailored therapies. The Cancer Genome Atlas (TCGA) project identified four distinct molecular subgroups in GBM. However, there was not

a significant correlation between patient survival and gene expression subtype (Verhaak et al., 2010; Noushmehr et al., 2010). The epigenetic G-CIMP phenotype was the only molecular classification predictive of survival (Noushmehr et al., 2010). Therefore, it was necessary to refine the molecular classification of GBM into subtypes with stronger prognosis value. Noticeably, long-term GBM survivors do not fall under a single molecular subtype and not necessarily all of them carry IDH mutations, but they are associated with a low mesenchymal gene signature (Gerber et al., 2014). Accordingly, a multi-cancer mesenchymal transition gene expression signature has been associated with shorter time to recurrence in GBM (Cheng et al., 2012; Kim et al., 2013). We have identified GPR56 as an important player in the PN to MES transition in GBM, which strengthens the idea that gene signatures associated with mesenchymal features may be clinically used to predict patient survival. The clinical predictive value in GBM of different MES signatures, including the GPR56 low activity-associated signature, highlights the great importance of understanding the molecular mechanisms that regulate the PN to MES transition. Furthermore, the GPR56 low activity-associated signature positively correlates with mesenchymal signatures in other tumor types, highlighting the importance of GPR56 in tumor progression beyond GBM. According to the GPR56 activity-associated gene expression, ovarian carcinoma (OV), breast invasive carcinoma (BRCA), kidney renal clear cell carcinoma (KIRC) and urothelial bladder carcinoma (BLCA) are the tumor types more similar to GBM regarding expression of the GPR56 low activity-associated signature. Interestingly, these tumors have also been classified into different subtypes, including a mesenchymal-like subtype. This suggests that GPR56 might also be playing an important role in tumor progression in these other tumor types (Prat et al., 2010; Brannon et al., 2010; Cancer Genome Atlas Research Network, 2013, 2011, 2014). Strikingly, GPR56 has been found downregulated in claudin-low breast tumors, which are associated with a poorer prognosis and display features of mesenchymal and inflammatory phenotypes (Prat et al., 2010). The GPR56 low activity-associated signature emerges as a robust set of markers to molecularly define MES-subtype tumors across different tumor types. Therefore, this signature might be used as a pan-cancer MES-subtype tumor signature.

In agreement with our results, *GPR56* is downregulated in highly metastatic melanoma cell lines (Xu et al., 2006), which might promote loss of cell adhesion and increase in cell motility. It would be interesting to test whether these

metastatic cell lines have a mesenchymal phenotype. Importantly, we have found that GPR56 low activity-associated signature positively correlates with MES signatures in other tumor types beyond GBM. This data suggests that GPR56 might have a general role as an inhibitor of the mesenchymal transition across multiple tumor types. Further studies addressing the function of GPR56 in other tumor types are required to test this hypothesis.

Mechanistically, we have identified GPR56 as a novel inhibitor of the NF- κ B signaling pathway, thereby providing a mechanism by which this receptor prevents the MES transition in GBM. We have shown that GPR56 inhibits canonical NF- κ B signaling by stabilizing I κ B α protein levels via inhibition of the IKK complex. However, the direct mechanism by which GPR56 inhibits IKK is still not well understood. The NF- κ B pathway is regarded as a promising candidate target for drug development in many cancer types (Prasad et al., 2010), including GBM, since there is a strong correlation between NF- κ B activation and gliomagenesis. Levels of NF- κ B activity are much higher in GBM compared to normal tissues and are associated with increasing grade in astrocytic tumors (Wang et al., 2004). In addition, several NF- κ B regulators have been found deregulated in GBM (Bredel et al., 2006; Atkinson et al., 2009). More recently, hyperactivation of NF- κ B signaling has been associated to MES-subtype GBM (Verhaak et al., 2010; Bhat et al., 2013). Therefore, identification of new major regulators of this pathway is of crucial interest to design effective therapies against MES-subtype GBM as well as other NF- κ B dependent tumors.

In this study we have also described that activated STAT3 inhibits *GPR56* expression. STAT3 has been described as one of the master regulators of mesenchymal transformation, which in GBM leads to the acquisition of important aggressive traits (Carro et al., 2010; Bhat et al., 2011). In an analogy between normal development and tumorigenesis, we observed negligible *GPR56* expression in both human and mouse ESC, while *GPR56* is upregulated during neural specification. Interestingly, STAT3 activation acts to sustain ESC self-renewal and pluripotency (Ying et al., 2003; Raz et al., 1999). Therefore, it would be interesting to test whether *GPR56* expression is also inhibited by STAT3 in the context of STAT3-mediated ESC maintenance.

Proneural GBM can undergo differentiation to a mesenchymal state in a TNF- α /NF- κ B-dependent manner via the induction of STAT3, C/EBP β and TAZ (Bhat et al., 2013). Therefore, we propose a model in which TNF- α activates both NF- κ B and STAT3 signaling, thereby promoting the transcriptional ac-

tivation of mesenchymal genes. At the same time, activated STAT3 represses GPR56 expression, thereby relieving NF- κ B inhibition by GPR56 and enhancing mesenchymal differentiation (Figure 5.2). Activation of NF- κ B and STAT3 pathways is a common feature of GBM and positively correlates with tumor grade (Lo et al., 2008). The crosstalk between NF- κ B and STAT3 pathways has been demonstrated at multiple levels, including activation of STAT3 by NF- κ B-regulated factors such as IL-6 and COX-2 (Naugler et al., 2007; Dalwadi et al., 2005). Constitutively activated STAT3 maintains constitutive NF- κ B activity in cancer by inhibiting its export from the nucleus (Lee et al., 2009). With this study, we show an alternative crosstalk between these two important pathways in which regulation of GPR56 defines a new cooperative association between NF- κ B and STAT3 and helps to explain why both transcription factors appear to stimulate a highly overlapping repertoire of tumorigenic genes in GBM. Additionally, our results may have important consequences in other fields beyond cancer biology, such as inflammation or innate immunity where NF- κ B signaling has a central regulatory role.

We have revealed a previously unknown STAT3-dependent regulation of *GPR56* expression. However, regulation of *GPR56* expression is still poorly understood. During embryonic development, different members of the regulatory factor X (RFX) transcription factor family promote *GPR56* expression in proliferative areas of the brain (Bae et al., 2014). In the context of GBM, RFX1 is a tumor suppressive transcription factor that acts via direct repression of CD44 expression (Feng et al., 2014). Therefore, the combined action of RFX1 on both GPR56 and CD44 expression might provide a double layer of regulation of the mesenchymal phenotype by RFX factors. Interestingly, the RFX1 gene is hypermethylated and silenced in GBM, thereby suggesting that RFX factors might have an important role in GBM pathogenesis and evolution (Ohashi et al., 2004).

Although alteration of GPR56 function has a profound effect on the PN to MES transition, *GPR56* genetic alterations are not commonly seen in MES-subtype GBM. Interestingly, *GPR56* promoter hypermethylation has been associated with acquired multidrug resistance in lung adenocarcinoma (Guo et al., 2013). Since transitions of stem cells into committed progenitors and eventually differentiated cells is a dynamic process mainly regulated by reversible epigenetic modifications, *GPR56* expression might also be regulated by epigenetic changes in GBM. Further studies on GPR56 epigenetic regulation are required for a

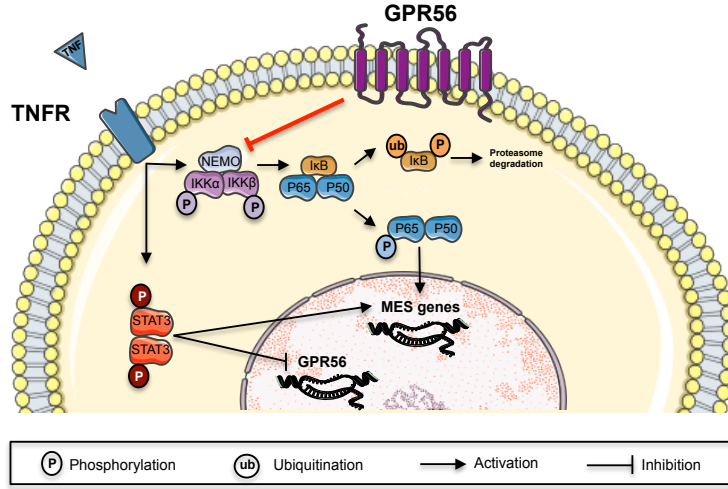


Figure 5.2: *Proposed molecular interactions between GPR56 and both NF- κ B and STAT3 signaling.*

In the basal state, GPR56 inhibits the NF- κ B signaling pathway upstream of NEMO. TNF- α activates both NF- κ B and STAT3 signaling, thereby promoting the transcriptional activation of mesenchymal genes. At the same time, activated STAT3 represses *GPR56* expression, thereby relieving NF- κ B inhibition by GPR56 and enhancing mesenchymal differentiation.

better understanding of the role of GPR56 in GBM biology.

5.2.3 GPR56 and the microenvironment

Cancer is often referred to as an ecological disease involving a dynamic interplay between malignant and non-malignant cells (Merlo et al., 2006; Lee et al., 2011). Tumor formation and progression involves the co-evolution of tumor cells together with their microenvironment. Therapeutic resistance often reflects active tumor evolution driven by the dynamic interplay between tumor cells and their surroundings when the selective pressure of therapy is applied. Therefore, tumor cell adaptation to microenvironment changes is one of the main hallmarks for success during tumor progression. GIC derived from MES-subtype GBM display a PN phenotype in culture, suggesting that tumor microenvironment factors play a pivotal role in tumor identity. Indeed, some factors that

promote changes in tumor microenvironment, such as inflammation or ionizing radiation (IR), have been identified as important regulators of the GBM phenotype (Halliday et al., 2014; Bhat et al., 2013). However, how tumor cells sense these changes remains unsolved. Here, we report how GPR56 controls the transition between different cellular states. Given the nature of GPR56 as an adhesion GPCR, which may sense changes in the microenvironment and convey these signals to the intracellular space, one might speculate that this receptor is one of the molecular links between the microenvironment and the cell's machinery regulating cell fate.

One crucial component of tumor microenvironment is the extracellular matrix (ECM), which actively regulates many aspects of tumor cell behavior (Joyce and Pollard, 2009). Increased density, elasticity and crosslinking of ECM are correlated with cancer malignancy (Colpaert et al., 2001; Reticker-Flynn et al., 2012). Strikingly, transglutaminase 2 (TG2), a crosslinking enzyme, has been described as a GPR56 ligand in melanoma. Although TG2 promotes melanoma growth through its crosslinking activity, GPR56 antagonizes this effect by internalizing and degrading it and, consequently, decreasing ECM deposition (Yang et al., 2014). Thus, it has been proposed that GPR56 inhibits melanoma metastasis via inhibition of TG2-mediated ECM remodeling. In GBM, TG2 is highly expressed in CD44^{high} GBM tissues and GIC. Inhibition of TG2 promotes a reduction in *ID1* expression, impaired cell proliferation and apoptosis (Fu et al., 2013). Additionally, high CD44 and ID1 levels have been associated with poor prognosis in GBM patients (Anido et al., 2010). Given the role of GPR56 in inhibiting CD44 expression in GBM and the antagonistic role of GPR56-TG2 in melanoma progression, further studies facing the association between GPR56 and TG2 would probably shed light into our understanding of GBM pathogenesis. However, the different levels of GPR56 regulation, such as many alternative splice and cleaved forms and multiple different interactions, makes understanding GPR56 function a great challenge ahead of us (Bae et al., 2014; Luo et al., 2014). Future studies regarding GPR56 interaction with the microenvironment are required for a better knowledge of GPR56 function during tumorigenesis.

The inflammatory component derived from microglia/macrophages plays a pivotal role during cancer progression. Glioma-infiltrating microglia/macrophages are present in GBM and their density is positively correlated with glioma grade and invasiveness (Markovic et al., 2005). Recent studies have shown that the MES subtype exhibits a higher degree of macrophages/microglia infiltration

(Cooper et al., 2012; Li and Graeber, 2012). A large number of evidences show that microglial cells are involved in creating a microenvironment that favors GBM progression (Zhai et al., 2011). For example, TNF- α derived from macrophages has been proposed to promote the transition from PN to MES states (Bhat et al., 2013). Therefore, a highly-vascularized microenvironment that favors immune cell infiltration might be an favourable niche during cancer progression. Indeed, GIC reside in close apposition to blood vessels (BV), also known as the perivascular niche (Calabrese et al., 2007). Some authors postulate that the intimate interplay between cancer cells and their surroundings is an important determinant for cancer progression (Barcellos-Hoff et al., 2013). Therefore, cancer cell niches can be considered potential targets for cancer prevention and therapy (Barcellos-Hoff et al., 2013). In this context, adhesion proteins play a pivotal role in keeping cancer cells localized to their niches and might as well serve as mediators that convey signals from the microenvironment to the cell. In this PhD thesis, we have shown that GPR56 mediates the adhesion of GIC to endothelial cells (EC). Interestingly, during embryonic development GPR56 mediates the attachment of radial glial endfeet to pial basement membrane (BM) (Koirala et al., 2009). Some of the components of the pial BM are also present in the extracellular matrix surrounding BV, such as laminin and collagen III. Collagen III is one of the main ligands for GPR56 (Luo et al., 2011) and cerebellar progenitors that lack GPR56 have an impaired adhesion to laminin and collagen III (Luo et al., 2011; Kim et al., 2009). Furthermore, during cerebral cortical development, synergistic activities of GPR56 and $\alpha 3\beta 1$ integrin, a laminin receptor, have been also demonstrated (Jeong et al., 2013). Taken together, our data suggest that GPR56 might play an important role in maintaining cancer stem-like cells attached to the perivascular niche and, at the same time, conveying signals from the microenvironment to the cell (Figure 5.3).

Furthermore, we have provided a novel mechanism by which some factors that promote tumor microenvironment changes, such as inflammation or IR, might promote a MES cascade: modulation of *GPR56* expression. We have shown that IR induces a reduction in GPR56 protein levels, thereby suggesting that the IR-induced MES shift might be due to, at least in part, GPR56 downregulation. Other MES inducers such as TNF- α also reduce GPR56 expression. Additionally, MES differentiation induced by TNF- α or IR is always reinforced by loss of GPR56. Therefore, IR might induce a positive loop that promotes the

conversion into a MES phenotype. IR directly promotes GPR56 downregulation through a cell-intrinsic mechanism, but it may also trigger an IR-associated inflammatory response, which might thereby enhance downregulation of GPR56 via TNF- α . Strikingly, GPR56 low activity-associated signature positively correlates with inflammatory signatures in different tumor types. These data shed light on how complex processes such as inflammatory tumor microenvironment or radiotherapy might promote EMT and the acquisition of therapy-resistant phenotypes. Since GPR56 levels inversely correlate with the MES phenotype and radioresistance, our studies might represent a starting point in predicting GBM response to radiotherapy and in developing patient-tailored therapies in GBM.

Taking all our results into account, we hypothesize that GPR56 may keep cancer stem-like cells in close apposition to the perivascular niche where they receive all the signals and factors necessary for tumor growth. However, during tumor progression there are changes in the microenvironment and cancer cells need to adapt and evolve to other cellular stages in order to survive. Here we show how microenvironment changes, such as inflammation or IR, might promote a MES shift by modulating *GPR56* expression. Therefore, it is plausible to think that while GPR56 is required for the adhesion of GIC to the perivascular niche in order to preserve their neural stem-like properties, during tumor progression loss of GPR56 is a crucial step leading to the acquisition of a mesenchymal phenotype (Figure 5.3).

5.2.4 Concluding remarks

In the present study, we have identified a differential *GPR56* expression between NSC and ESC/MSC, whereby *GPR56* is upregulated during the course of ESC differentiation into neural lineages. Analogously, *GPR56* is enriched in PN-subtype GBM and a decrease in *GPR56* activity promotes MES differentiation, radioresistance and poor prognosis in GBM. Therefore, we have described for the first time that an adhesion GPCR controls the transition between different cellular states. Given the lack of E-cadherin expression in normal brain and GBM (Lewis-Tuffin et al., 2010), the identification of GPR56 as novel negative regulator of EMT represents an important step forward in the GBM field. GPR56

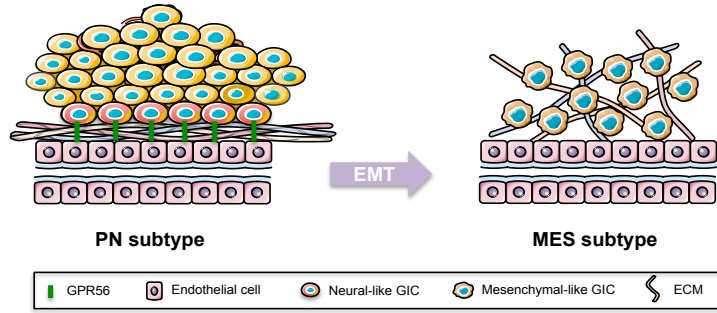


Figure 5.3: *Model of GPR56 function during GBM progression.*

GPR56 mediates the attachment of GIC to the perivascular niche, thereby preserving their neural stem-like phenotype. During GBM progression from PN to MES subtype, loss of GPR56 results in the detachment of GIC from EC leading to the acquisition of a mesenchymal phenotype.

is an adhesion receptor that mediates cell-to-cell and cell-to-matrix interactions (Koirala et al., 2009; Luo et al., 2014). Therefore, it would be plausible to propose that GPR56 is a lineage specification factor that restricts ESC cell potential by sensing the microenvironment and sending a message to the cell to acquire a more restricted fate given the proper environmental conditions. At the same time, GPR56 regulation would provide the means for adapting cell fate to changes in the microenvironment in both stem and cancer cells.

Furthermore, we have identified GPR56 as a novel inhibitor of the NF- κ B signaling pathway, providing a mechanism by which this receptor prevents the MES transition. Since *GPR56* levels inversely correlate with the MES phenotype and radioresistance, these studies represent a starting point in predicting GBM response to radiotherapy and in developing personalized therapies.

6

Conclusions

1. Transcriptional profiling of hypoxic NSC identifies calcineurin-NFATc4 signaling as a major regulator of NSC biology:

- ❖ Physiological hypoxia induces large changes in the transcriptome of NSC, thereby promoting self-renewal and proliferation.
- ❖ Physiological hypoxia promotes a switch to a non-oxidative metabolism in NSC as well as the activation of the calcium signaling pathway.
- ❖ Calcium-activated calcineurin signaling promotes self-renewal and proliferation of hypoxic NSC.
- ❖ GSEA identifies *Nfatc4* as a major regulator of hypoxic NSC functions.
- ❖ Physiological hypoxia promotes *Nfatc4* upregulation at the transcriptional level.
- ❖ NFATc4 promotes the expression of TF that regulate the maintenance of the NSC state, such as *Id2* and *FoxP2*.
- ❖ NFATc4 promotes NSC self-renewal and proliferation *in vitro* and *in vivo*.

Conclusions

2. GPR56 is a NSC factor that restricts the proneural to mesenchymal transition in GBM by inhibiting the NF- κ B pathway:

- ❖ *GPR56* is progressively upregulated in human ESC that undergo differentiation into the neural lineage.
- ❖ In the adult mouse brain, GPR56 protein is expressed in Sox2⁺ neural progenitors that closely contact BV in the SVZ.
- ❖ In GBM, GPR56 is enriched in PN-subtype tumors whereas the MES-subtype tumors express low levels of *GPR56*. *GPR56* expression positively correlates with well-established PN signature genes.
- ❖ Most GIC display PN-subtype features in cell culture. TNF- α induces an increase in MES gene expression without a reduction in PN gene expression.
- ❖ *GPR56* is highly expressed in PN GIC whereas it is poorly expressed in MES GIC. CD44^{low} GIC express high levels of *GPR56* while CD44^{high} GIC show poor *GPR56* expression.
- ❖ GPR56 promotes the adhesion of GIC to EC thereby maintaining GIC attached to their perivascular niche.
- ❖ TNF- α or IR promote a decrease in *GPR56* expression. IR- and TNF- α -induced MES differentiation is enhanced in *GPR56*-knockdown GIC, suggesting that downregulation of GPR56 is required for MES differentiation.
- ❖ GPR56 prevents the acquisition of a global MES differentiation program in GIC both *in vitro* and in mouse orthotopic xenograft models. Analogously to TNF- α treatment, loss of GPR56 is not able to trigger a global reduction in PN genes.
- ❖ GPR56 inhibits properties associated with a mesenchymal phenotype such as loss of cell adhesion and radioresistance.
- ❖ Low GPR56 activity is a poor prognosis factor in GBM patients. Patients with high expression of genes within a GPR56 low activity-associated signature have decreased survival.

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- ❖ GPR56 inhibits NF- κ B signaling by reducing NEMO protein levels and thereby stabilizing I κ B α protein.
 - ❖ Activated STAT3 inhibits GPR56 expression, thereby contributing to enhance NF- κ B signaling.
 - ❖ We proposed a model in which TNF- α activates STAT3, thereby decreasing GPR56 expression and, consequently, relieving NF- κ B pathway inhibition and promoting MES differentiation.
 - ❖ A pan-cancer analysis of GPR56 low activity-associated signature reveals that GPR56 activity inversely correlates with EMT and inflammation signatures while it positively correlates with epithelial signatures in different tumor types beyond GBM. Therefore, the GPR56 low activity-associated signature might be used as a pan-cancer MES-subtype tumor signature.

Conclusions

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