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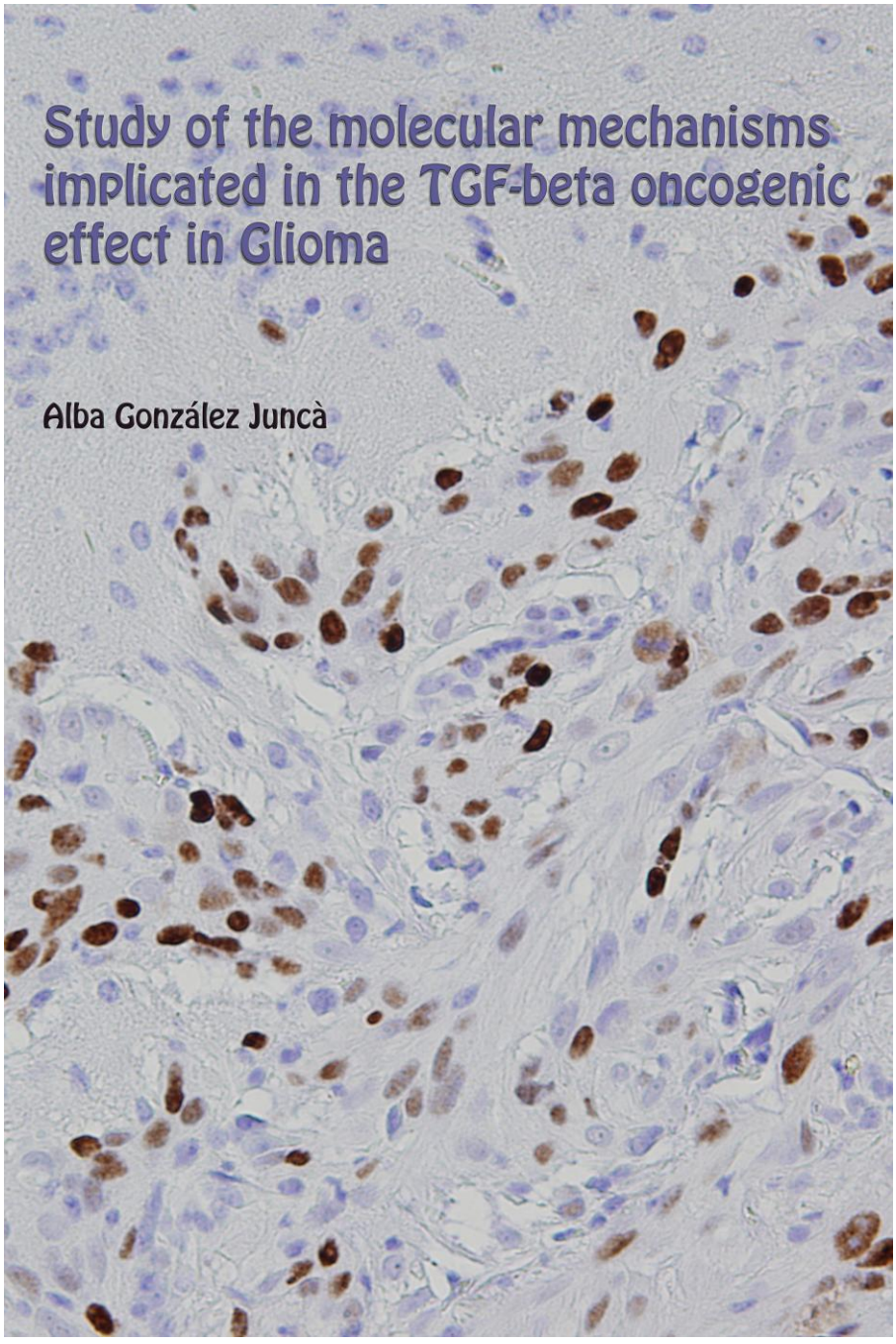

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STUDY OF THE MOLECULAR MECHANISMS IMPLICATED IN THE TGF-BETA ONCOGENIC EFFECT IN GLIOMA

ESTUDI DELS MECANISMES MOLECULARS IMPLICATS EN L'EFECTE ONCOGÈNIC DEL TGF-BETA EN GLIOMA

Memòria persentada per **ALBA GONZÁLEZ JUNCÀ** per optar al grau de doctor/a per la Universitat de Barcelona

ALBA GONZÁLEZ JUNCÀ JOAN SEOANE SUÁREZ (Director)

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STUDY OF THE MOLECULAR MECHANISMS IMPLICATED IN THE TGF-BETA ONCOGENIC EFFECT IN GLIOMA

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LIST OF ABREVIATIONS

ABREVIATIONS

AMH	Anti-Müllerian Hormone
AML	Acute Mieloid Leukemia
ANGPTL-4	Angiopoietin-like 4
BCAN	Brevican
bHLH	basic-Helix-Loop-Helix
BMP	Bone Morphogenic Protein
bp	Base pairs
BrdU	Bromo-deoxi-Uridine
BrET	Ethidium Bromide
BV	Blood Vessel
C.M.	Conditioned Media
CBF	Core Binding Factor
CDK	Cyclin Dependent Kinase
ChIP	Chromatin Imunoprecipitation
CIC	Cancer Initiating Cell
CNS	Central Nervous System
CNV	Copy Number Variation
CSC	Cancer Stem Cell
Da	Dalton
DAB	Diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dymethil Sulfoxide

DNA	Desoxiribonucleic Acid
ECL	Enhanced Chemiluminescent substrate Luminol-based
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epithelial to Mesenchymal Transition
ESC	Embryonic Stem Cell
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug administration
FFPE	Formalin-Fixed Paraffin-Embedded
FGF	Fibroblast Growth Factor
FISH	Fluorescence *In Situ* Hybridization
FN	Fibronectin
GBM	Glioblastoma
GEMM	Genetically Engineered Mouse Models
GF	Growth Factor
GFAP	Glial Fibrillary Acidic Protein
GIC	Glioma Initiating Cell
GSC	Glioma Stem Cell
H&E	Hematoxilin & Eosin
HCl	Clohridric Acid

hCMEC	human Cerebral Microcapilar Endothelial Cells
HDACs	Histone Deacetylases
HIER	Heat-Induced Epitope Retrieval
HRP	Horseradish Peroxidase
HUVEC	Human Umbilical Vein Cord
IB	Immunoblott
ID	Inhibitor of Differentiation
IF	Immunofluorescence
Ig	Immunoglobulin
IHC	Immunohistochemistry
JAK	Janus Kinase
K.O.	Knock-Out
KDa	Kdalton
LB	Lysogeny Broth
LCM	Laser-assisted Capture Microdissection
LIF	Leukemia Inhibitory Factor
MAPK	Mitogen-Activated Protein Kinase
MEF	Mouse Embryonic Fibroblasts
MHC	Major Histocompatibility Complex
miRNA	micro RNA
MLV	Murine Leukemia Virus
MMP	Matrix Metallo-Protease
MRI	Magnetic Resonance Image

mRNA	messenger RNA
Msh-1	Musashi-1
mTOR	mamalian Target of Rapamycin
NaCMC	Sodium Metil-Cellulose
NF1	Neurofibromatosis Factor 1
NF-KB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NGS	Next Generation Sequencing
NK	Natural Killer
NOD/SCID	Non-Obese Diabetic/ Severe Combined Immuno Deficiency
NSC	Neural Stem Cell
NSPH	Neurosphere
NTRKA	Neurotrophic Tyrosin Kinase Receptor A
O/N	Over-night
PAI-1	Plasminogen Activator Inhibitor 1
PBS	Phosfate Buffered Saline
PCR	Polymerase Chain Reaction
PCTC	Primary Culture Tumor Cells
PI3K	Phophatidyl-Inositol 3-Phosphate Kinase
PKC	Protein Kinase C
PN	Proneural
pS6	phospho-S6 kinase
p-Smad2	phospho-Smad2
PVDF	Polyvinylidene difluoride

qRT-PCR	quantiative Real Time Polymerase Chain Reaction
RB	Retinoblastoma
REMBRANDT	REpository for Molecular BRAin Neoplasia DaTa
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
R-Smads	Receptor-activated Smads
RTK	Receptor-associated Tyrosine Kinases
RUNX	Runt-related transcription factors
RT	Room Temperature
SBE	Smad Binding Element
SD	Standard Deviation
Shh	Sonic Hedgehog
shRNA	short hairpin RNA
siRNA	small interference RNA
SMA	Smooth Muscle Actin
STAT	Signal Transducer and Activator of Transcription
TBS	Tris-buffered Saline
TBV	Tumor Blood Vessel
TCGA	The Cancer Genome Atlas
TE	Tris-EDTA
TF	Transcription Factor
TFBS	Transcription Factor Binding Site

TGFβ	Transforming Growth Factor beta
TMA	Tissue Microarray
TNF	Tumor Necrosis Factor
Treg	T-Regulatory limphocytes
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TβRI	TGFβ Receptor I
TβRII	TGFβ Receptor II
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
WT	Wild Type

INTRODUCTION

1. GLIOMA

Gliomas are a group of tumors located in the brain. The name derives from a cellular resemblance to glia, cells that provide mechanical support and an inflammatory response and maintain homeostasis in the Central Nervous System (CNS) (Figure 1.1) (Mamelak and Jacoby 2007). Gliomas account for 30% of all brain and CNS tumors and 80% of all malignant brain tumors (Goodenberger and Jenkins 2012).

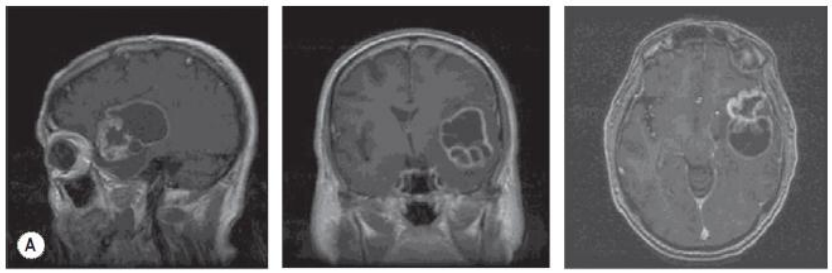


Figure 1.1. Different images of glioblastoma (GBM). Glioma is a tumor located in the brain. In these MRI images it can be observed as a mass growing in the right cortex with some necrotic areas in the centre. Images from Kaye and Laws, Brain Tumors: an encyclopedic approach, 2011.

HISTOPATHOLOGICAL CLASSIFICATION

Gliomas can be classified according to histology and the predominant cellular morphology. Main glioma types include:

- Astrocytomas: astrocytes
- Ependymomas: ependymal cells
- Oligodendrogliomas: oligodendrocytes
- Mixed gliomas: oligoastrocytomas

As in other tumor types, the World Health Organization (WHO) determines different grades of gliomas according to the pathologic evaluation of the tumor (Figure 1.2) (Wrensch, Rice et al. 2006; Fuller and Scheithauer 2007; Sulman, Guerrero et al. 2009).

- **Low grade gliomas** (WHO grade II): not anaplastic and well differentiated gliomas. They have a better prognosis. Includes diffuse astrocytoma.
- **High grade gliomas** (WHO grade III-IV): anaplastic and poorly differentiated gliomas. They have a worse prognosis. Includes grade III anaplastic astrocytoma (AA) and grade IV astrocytoma, also called glioblastoma (GBM).

  Glioblastoma (GBM) is the most malignant form of glioma (grade IV) and it represents 20% of brain tumors with an incidence of 3 in 100,000 per year (Goodenberger and Jenkins 2012). Although it is a non-metatasizing tumor, GBM cells are highly invasive throughout the brain, leading to the destruction of normal brain tissue. Furthermore, it is resistant to conventional therapies (radio and chemotherapy) and highly deadly (Furnari, Fenton et al. 2007; Kotliarova and Fine 2012).

Histologically, glioma presents nuclear atypia, hyperproliferation (high number of mitosis), necrosis and/or endothelial proliferation (Figure 1.2).

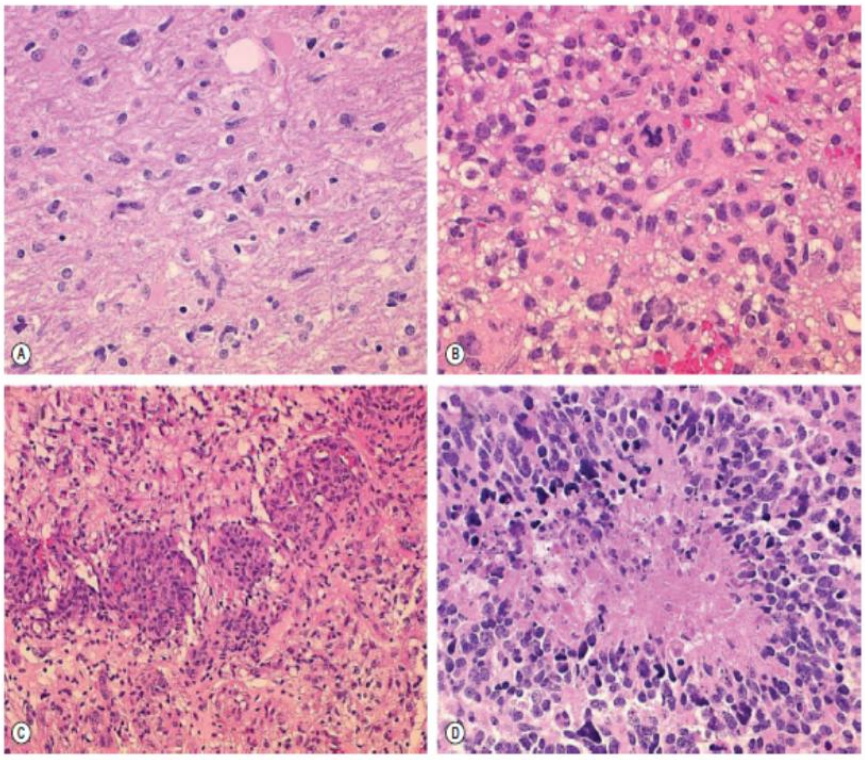


Figure 1.2. Histologic features of astrocytomas. **A**. Fibrillary astrocytoma (WHO grade II) with pleomorphic astrocytes and increased cellularity. **B.** Anaplastic astrocytoma (WHO grade III) with increased cellularity, nuclear polymorphism and mitosis. **C.** Glioblastoma (WHO grade IV) with microvascular proliferation. **D.** Glioblastoma (WHO grade IV) with nuclear pleomorphism and necrosis. Hematoxilin & Eosin staining, A, B, D: 400x magnification. C: 200x magnification. Images from Kaye and Laws, Brain Tumors: an encyclopedic approach 2011.

PROGNOSIS

Gliomas are rarely curable. The prognosis for high-grade glioma patients is generally poor. Prognosis depends on different factors such as the

patient's age, location of the tumor and extent of the resection. Grade III astrocytoma patients typically have 2-3 year survival, whereas GBM has the worst prognosis with a median survival of only 15 months despite the advances in treatments (Stupp, Mason et al. 2005).

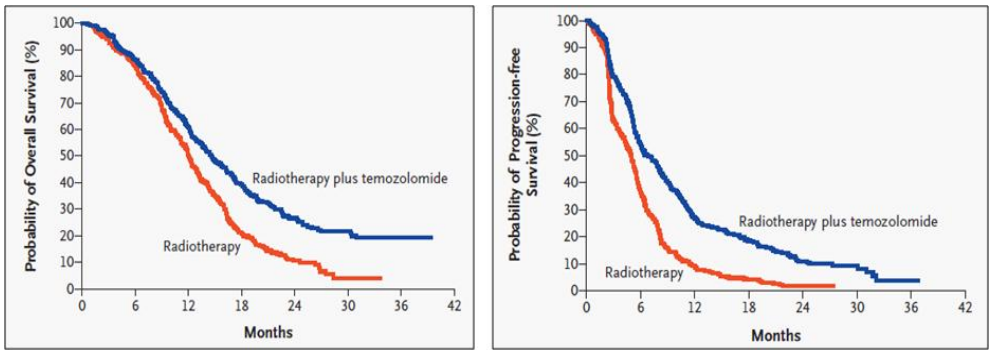


Figure 1.3. Kaplan-Meier survival plot of GBM patients treated with Radiotherapy alone or radiotherapy combined with temozolamide. Overall survival and progression-free survival were significantly increased when temozolamide was administrated concomitantly with radiotherapy ($p<0.001$). From (Stupp, Mason et al. 2005).

STANDARD OF CARE THERAPY FOR GLIOMA

Treatment of glioma depends on the tumor location and malignancy. The most used approach combines neurosurgery and radiotherapy together with chemotherapy (Figure 1.3) (Stupp, Mason et al. 2005). Standard therapy has been relatively ineffective for several reasons: first of all, the high invasive capacity of GBM cells into normal brain tissue limits the extent of surgical resection and high dose radiotherapy without permanent neurological damage to the patient (Kotliarova and Fine 2012). It has been shown that the extent of the resection measured by post-operative MRI

correlates with a better outcome, including progression-free survival and overall patient survival (Lacroix, Abi-Said et al. 2001; Sanai and Berger 2008). Once the surgical phase is complete, viable tumor cells remain in the brain parenchyma and so chemotherapy and radiation therapy are still needed. Radiotherapy has been a standard of care for patients with malignant glioma (Buatti, Ryken et al. 2008). Protocols usually prescribe a 60 Gy treatment in 2 Gy daily doses over a period of 6 weeks (Laperriere, Zuraw et al. 2002). Temozolamide was approved for treatment of astrocytomas in 2005 (Stupp, Hegi et al. 2009). Temozolamide is an alkylating agent that can effectively cross the blood-brain barrier, hence its use in the management of GBM. The Food and Drug Administration (FDA) also approved the use of Gliadel implantable Carmustine (BCNU) wafers (Attenello, Mukherjee et al. 2008). Despite treatment, tumor recurrence almost always occurs. Although both show an increased survival compared to placebo, these agents are ineffective in the treatment of recurrent gliomas (Lacroix, Abi-Said et al. 2001; Westphal, Ram et al. 2006; Chen, McKay et al. 2012). Hence a need for new therapies based on the molecular alterations that drive gliomagenesis.

MAIN CHARACTERISTICS OF GBM

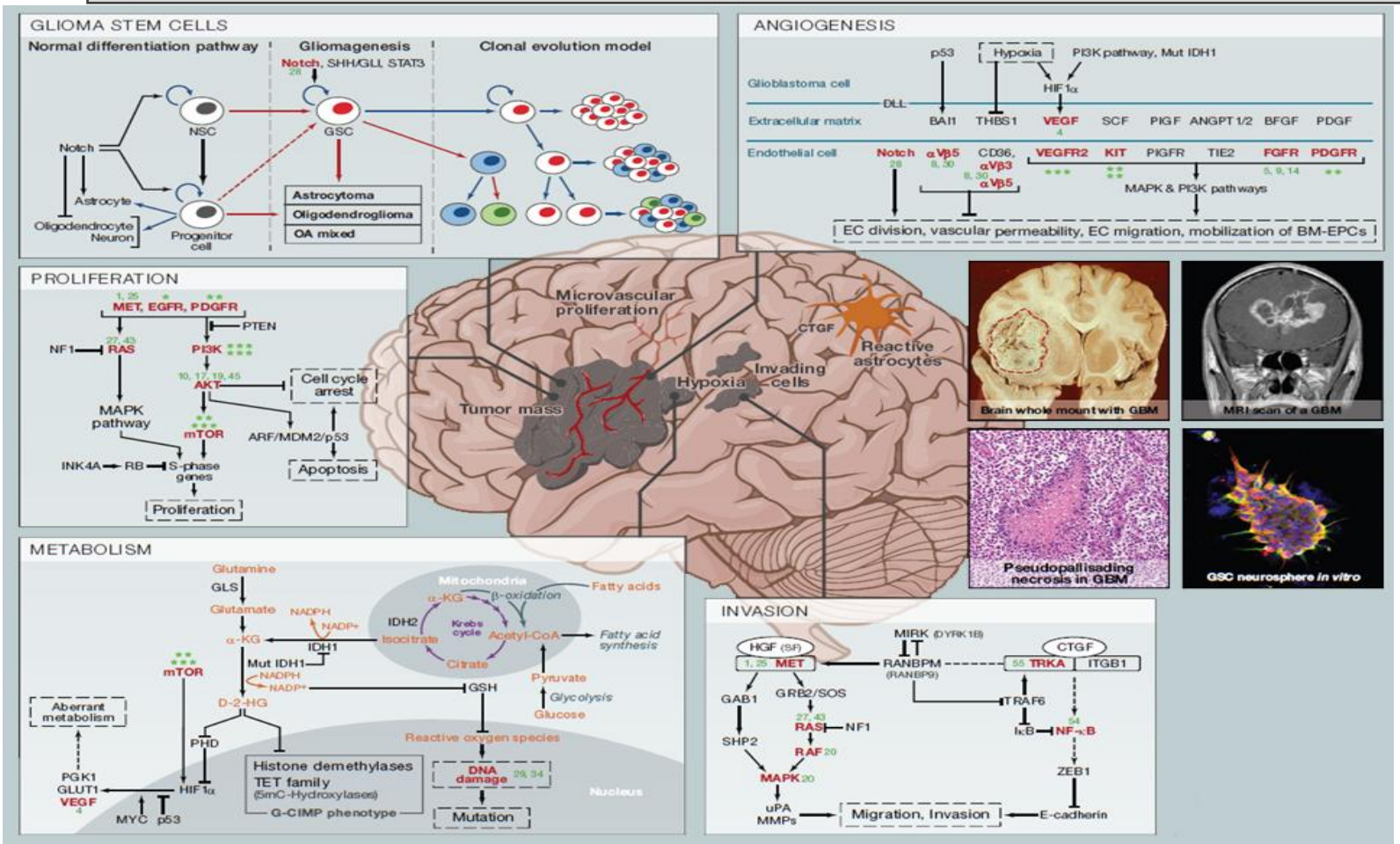
GBM is highly malignant, mainly because of the following properties, summarized in Figure 1.4 (Kotliarova and Fine 2012):

- **Proliferation**: GBM is a highly proliferative tumor, in part because it has cell-cycle deregulation, mainly due to abnormal signaling of Receptor-associated Tyrosine Kinases (RTKs) including Epidermal

Growth Factor Receptor (EGFR), Platelet-Derived Growth Factor Receptor (PDGFR) and MET. These RTKs activate downstream pathways such as the Mitogen-Activated Protein Kinase (MAPK) pathway or Phosphatidyl-Inositol 3 Phosphate- Kinase (PI3K) pathway. There are also deregulations in cell-cycle controlling proteins, such as the loss of $p14^{ARF}$ and $p16^{INK4A}$ as well as inactivation of *CDKN2B* and *TP53*. Loss or inactivation of *PTEN* and *NF1* are also frequent, which lead to a hyperactivation of PI3K and Ras-MAPK pathways respectively.

- **Metabolism**: GBMs, like other tumors, have an altered glucose metabolism, a phenomenon known as the Warburg effect, by which tumor cells produce energy at a high rate of glycolysis followed by lactic acid fermentation in an aerobic process. (Ponisovskiy 2010; Upadhyay, Samal et al. 2012). This altered metabolism leads to a dependency on altered glucose and fatty acid metabolism and a generation of excess reactive oxygen species (ROS).
- **Angiogenesis**: GBMs are highly angiogenic and vasculogenic. Vascular Endothelial Growth Factor (VEGF) is the main mediator of tumor angiogenesis. Due to its rapid growth, gliomas are very dependent on angiogenesis, this is why there are several clinical trials with anti-angiogenic therapies for GBM patients (Gerstner, Duda et al. 2007).
- **Invasion**: One of the main characteristics of GBM is that it is highly invasive. Cancer cells migrate throughout the normal brain, causing the destruction of brain parenchyma that is the most frequent cause of death in GBM. PI3K and MAPK pathway deregulation has been linked with increased cellular motility, especially via EGFR signaling (Zohrabian, Forzani et al. 2009; Feng, Hu et al. 2013). Amplification and overexpression of HGF/MET pathway have also been related to GBM invasion (Wang, Le et al. 2003; Eckerich, Zapf et al. 2007).

Figure 1.4. Main characteristics of GBM. GBM presents as a tumor mass in the brain, that is highly invasive, hypoxic and with microvascular proliferation. It is very proliferative and angiogenic and with an aerobic metabolism. It is thought that the cell of origin of GBM are Glioma Initiating Cells (GICs) which share some characteristics with normal neural stem cells and have the capacity to initiate a tumor. Here are summarized the main pathways and proteins involved in each of these features of GBM. From (Kotliarova and Fine 2012).



GENETIC CLASSIFICATION OF GBM

Based on genetic analysis data, GBM can be divided in two types: **primary** or *de novo* GBM and **secondary** GBM. Primary GBM typically affect older individuals (after 50 years old), have a short presentation and arises with no evidence of low grade lesions such as diffuse astrocytoma or anaplastic astrocytoma. In contrast, secondary GBMs affect younger individuals (less than 45 years old), with a prior malignancy that further progresses to GBM. Both types of tumors reach the malignant phenotype of GBM through distinct genetic pathways (Figure 1.4). In primary GBMs, EGFR is typically amplified or overexpressed (Ekstrand, Sugawa et al. 1992). They also present alterations and mutations in the p53 pathway, such as mutations in the MDM2 gene (Biernat, Debiec-Rychter et al. 1997). In contrast, secondary GBMs are characterized by a high frequency of mutation in p53 (Watanabe, Sato et al. 1997) and amplification or overexpression of PDGF-R (Hermanson, Funa et al. 1992).

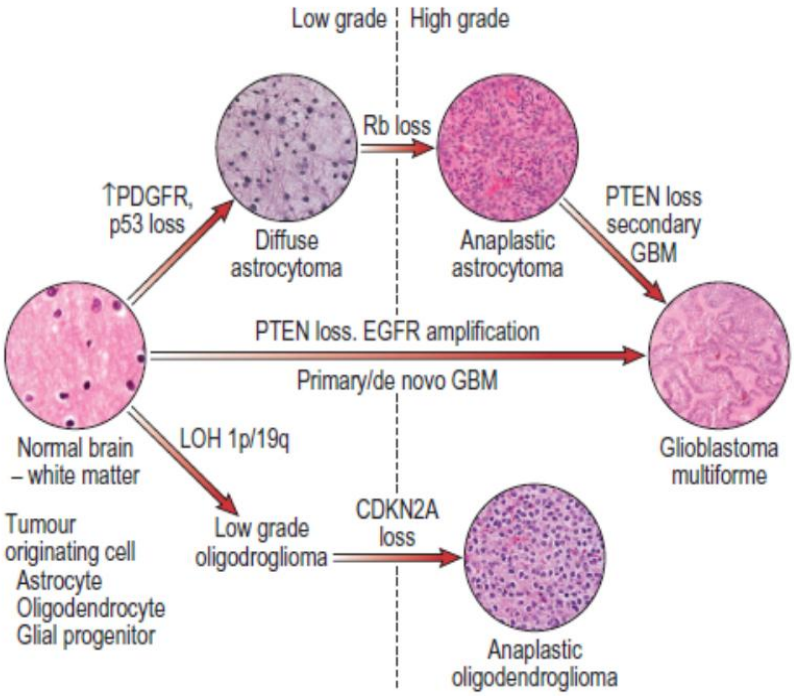


Figure 1.5. Genetic alterations in glioma progression. Low grade and high grade gliomas differ not only in their characteristics but also in the genetic alterations. Also primary (or *de novo*) GBM and secondary GBM have some differences in their genetic mutations. Images from Kaye and Laws, Brain Tumors: an encyclopedic approach 2011.

GBM MOLECULAR ALTERATIONS

Besides the genetic alterations, GBMs are characterized by aberrant signaling of different Growth Factor Receptors. Growth Factors (GFs) function as paracrine and autocrine signals to increase growth and proliferation of tumor cells. The most common abnormalities in GF signaling in GBM are secretion of VEGF, PDGF, Transfroming Growth Factor beta (TGFβ) and HGF (Hoelzinger, Demuth et al. 2007). It has also been wildely studied the amplification of Epidermal Growth Factor Receptor (EGFR), or the constitutively active mutated form (EGFRvIII), both accounting up to 45% of gliomas (Chakravarti, Dicker et al. 2004). GF stimulation or hyperactivation of receptors (RTKs) leads to increased signaling through Ras/Mitogen Activated Protein Kinase (MAPK) and phosphatidyl-inositol 3 kinase (PI3K) pathways. The result of hyperactivation of these pathways is a selective growth/proliferation advantage for tumor cells.

There are also alterations in cell cycle control in GBM. The most typical is the loss of $p14^{ARF}$ and $p16^{INK4A}$ due to the deletion of the locus that encodes both genes, CDKN2A, which occurs in almost 50-60% of GBMs. Inactivation of CDKN2B, amplification of cyclin-dependent kinases (CDK) 4 and 6 and p53 are also important steps in gliomagenesis. In the case of p53, mutations or homozygous deletion occurs in 30-60% of GBM (Figure 1.5) (Rao, Uhm et al. 2003; Parsons, Jones et al. 2008; Mao, Lebrun et al. 2012), (The Cancer Genome Atlas (TCGA), 2008).

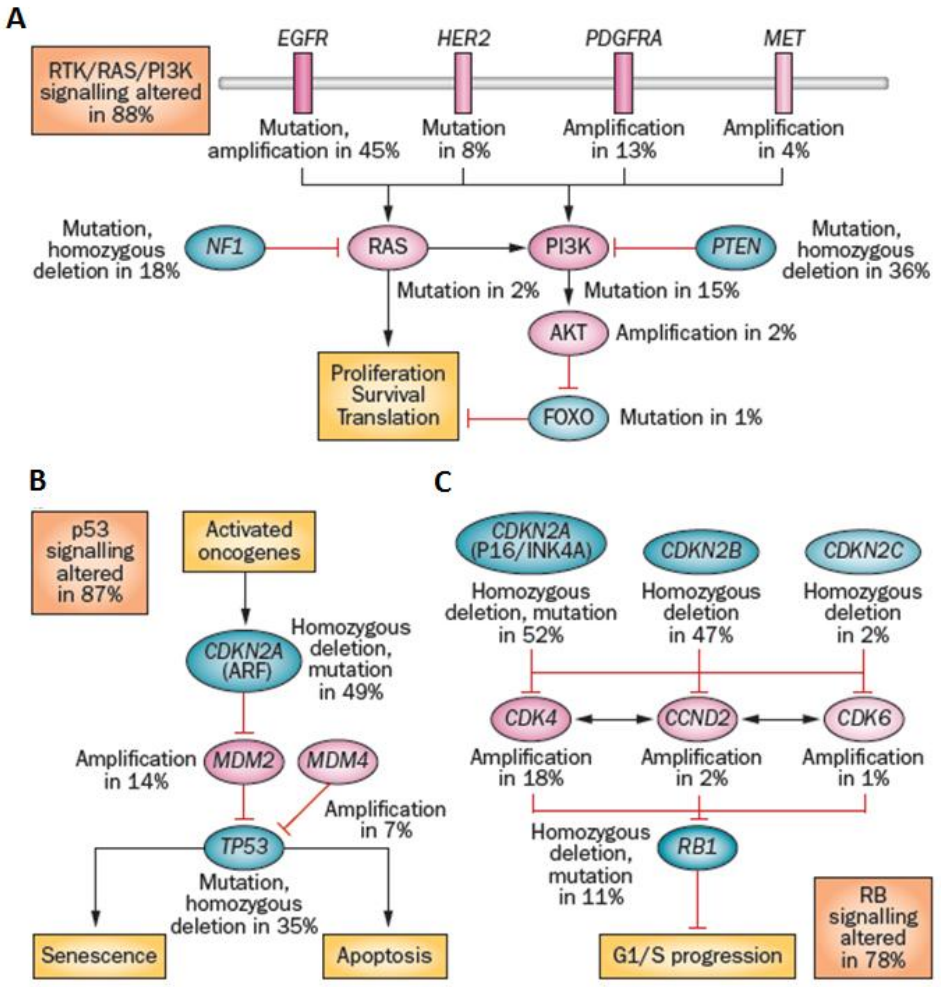


Figure 1.6. The most common mutations in GBM. The most frequently mutated pathways in GBM are RTKs (RAS/PI3K) signaling pathways (**A**) altered in 86% of GBM patients, p53 pathway (**B**) altered in 87% of GBM patients and RB signaling pathway (**C**), mutated in 78% of GBM patients. In **purple** are amplifications or mutations leading to hyperactivation, and in **blue** deletions or inactivating mutations. Adpted from the The Cancer Genome Atlas Research Network 2008, extracted from (Parsons, Jones et al. 2008; Tanaka, Louis et al. 2013).

GBM SUBTYPES

Glioma is a very heterogeneous tumor that has been recently subdivided into 4 different groups according to genetic and chromosomic alterations: **Classical**, **Proneural**, **Neural** and **Mesenchymal** (Figure 1.6) (The Cancer Genome Atlas (TCGA), 2008), (Verhaak, Hoadley et al. 2010). Gene expression profiling and copy number alteration analysis has been performed to discern the molecular characteristics of those subgroups (Nutt, Mani et al. 2003; Liang, Diehn et al. 2005; Nigro, Misra et al. 2005; Phillips, Kharbanda et al. 2006; Parsons, Jones et al. 2008; Verhaak, Hoadley et al. 2010).

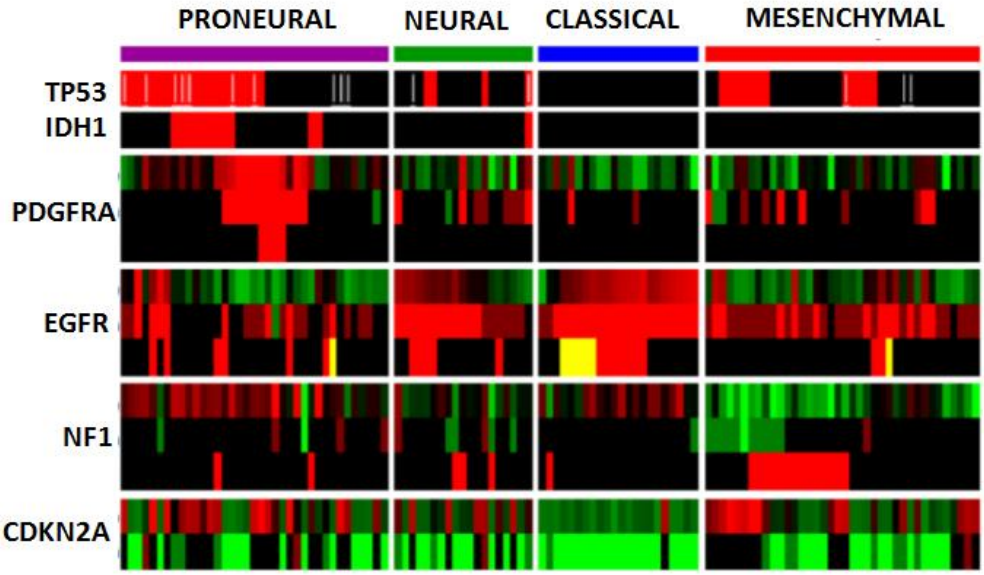


Figure 1.7. Main GBM subtypes according to its Copy Number Variation (CNV) and gene expression. GBM can be subclassified into 4 different subtypes: **Proneural**, characterized by PDGFRA amplification; **Classical**, characterized by EGFR amplification; **Mesenchymal**, characterized by NF1 loss and **Neural**, similar to classical but with expression of neuronal lineage markers. Adapted from (Verhaak, Hoadley et al. 2010)

The **Classical** subtype is characterized by chromosome 7 amplification, CDKN2A deletion, chromosome 10 loss, EGFR amplification or mutation, lack of *TP53* mutations and RB pathway alterations. Cells highly express Nestin and have hyperactivation of Notch and Hedgehog pathways (Verhaak, Hoadley et al. 2010).

Mesenchymal subtype patients present the worst prognosis within all the groups of GBM (Phillips, Kharbanda et al. 2006). Typically there are frequent inactivating mutations or loss of NF1, *TP53* and PTEN. There are also frequent chromosomal aberrations in CDK6, MET, PTEN, CDKN2A and RB1 loci. Tumors are highly malignant with expression of MET, CD44, and CHI3L1 (also known as YKL-40) (Tanwar, Gilbert et al. 2002; Nutt, Betensky et al. 2005; Pelloski, Mahajan et al. 2005; Bhat, Pelloski et al. 2008). Typically, mesenchymal tumor cells present hyperactivation of NFκB and TNF pathways (Brennan, Momota et al. 2009; Lee, Ramakrishnan et al. 2013)

Microarray differential gene expression of GBM subtypes, shows overexpression of different mesenchymal and neural stem-cell associated genes such as TNC, FN1, Sox2, Sox4, NES, VEGF, IGFBP5, MMP9, DLL3, *ID3*, CD44 (Phillips, Kharbanda et al. 2006; Tso, Shintaku et al. 2006).

A transcriptional network that regulates this mesenchymal phenotype has been recently described. Bioinformatical analysis of the promoter region of genes differentially expressed in mesenchymal tumors has revealed a gene signature of six Transcription Factors (TFs) that are responsible for regulating the mesenchymal transformation (Carro, Lim et al. 2010). These transcription factors are: C/EBPβ, STAT3, Runx1, bHLHB2, FOSL2 and ZNF238. This six transcription factors are thought to regulate all the genes

that are differentially expressed in mesenchymal GBMs, and as such are the master regulators of this subtype of GBM (Figure 1. 7).

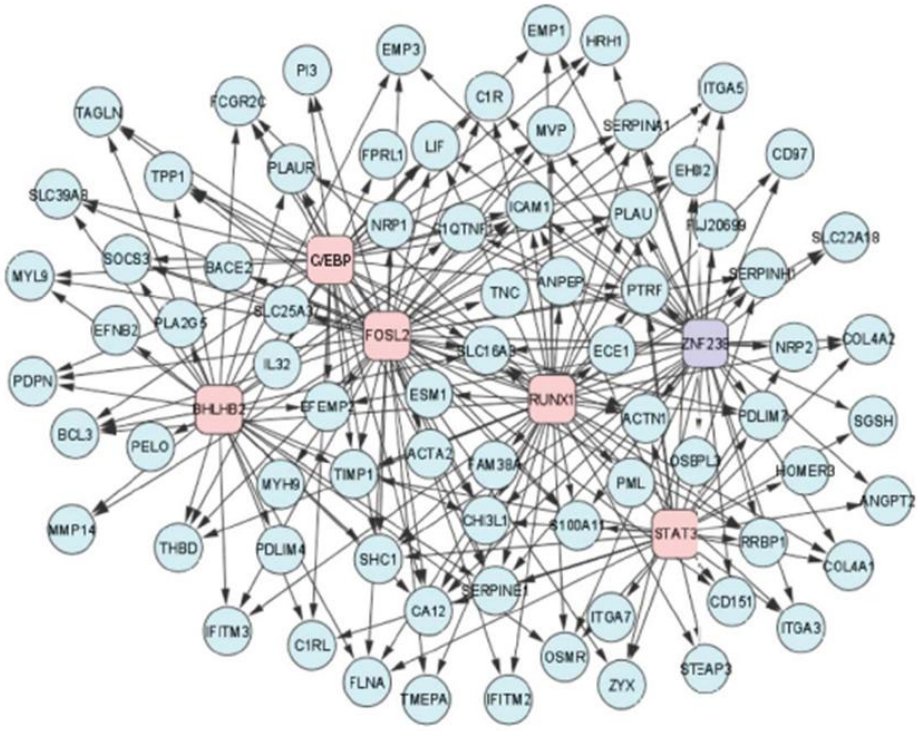


Figure 1.8. Transcriptional network for the mesenchymal subtype of GBM. Schematic representation of the genes differentially expressed in mesenchymal GBMs. In squares, the six transcription factors that regulate all the genes. C/EBP, BHLH-B2, FOSL2, RUNX1 and STAT3 (**in pink**) are activators of transcription whereas ZNF238 (**purple**) is a negative regulator of transcription. From (Carro, Lim et al. 2010).

Proneural (PN) subtype tumors have frequent mutations in IDH1 or IDH2 together with PDGFR or PDGFA amplifications or mutations and *PIK3*CA mutations. There is a loss of *TP53*, CDKN2A and PTEN. HIF, PI3K and PDGFR pathways are hyperactive. Tumors are characterized by high expression of

Olig-2, NKX2.2, PDGFRα, TCF4, SOX, DCX, DLL3 and ASCL1 markers. Within PN tumors there is a distinct subgroup characterized by a hypermethylator phenotype presenting IDH1 and 2 mutations, with better clinical outcome (Yan, Parsons et al. 2009; Lu, Ward et al. 2012; Turcan, Rohle et al. 2012).

Neural subtype is related to the classical subtype but with higher frequency of *TP53* mutation, EGFR amplification or overexpression and expression of different neuronal markers (NEFL, GABRA1, SYT1 and SLC12A5). Some unpublished results from our group and others suggest that the neural subtype may be an artifact of normal brain contamination when profiling studies are performed.

TARGETED THERAPIES AND CLINICAL TRIALS FOR GLIOBLASTOMA

Despite the standard treatment with resection, radiation and chemotherapy, glioblastoma patients' prognosis remains poor. The increasing knowledge of molecular alterations that drive glioblastoma progression has lead to the development of novel targeted therapies (Tanaka, Louis et al. 2013). Nowadays there are several clinical trials for GBM patients using novel targeted drugs. Although first generation targeted agents such as anti-EGFR therapies have not been as effective as expected, recent improvements in target identification, drug development, clinical trial design and patient selection for specific therapies promise some advances in the treatment of glioblastoma patients.

Targeted therapies are based in the molecular alterations that drive the gliomagenesis, listed in the previous sections and summarized in figure 1.6 (Parsons, Jones et al. 2008; Tanaka, Louis et al. 2013).

EGFR-targeted therapies

EGFR tyrosine kinase inhibitors erlotinib and gefitinib were the first targeted agents to be tested in glioblastoma patients as a monotherapy or in combination with standard of care treatment. They did not show any significant benefit. Neither treatment improvement has been shown with cetuximab, a monoclonal antibody against EGFR. Nowadays, there are next-generation EGFR TKIs with an irreversible EGFR inhibition that are in clinical trials for glioblastoma, such as afatinib, dacomitinib and nimotuzumab (an anti-EGFR humanized antibody).

PI3K-mTOR inhibitors

Whereas mTOR antagonists such as temsirolimus and everolimus have been tested in clinical trials for GBM showing minimal activity and no overall survival benefit, it has been suggested that mTOR inhibitors may be effective in a subpopulation of GBM patients with high levels of phosphorilation of ribosomal S6 kinase, a downstream activatior of mTOR signaling (Kreisl, Lassman et al. 2009). New agents are under clinical trials for recurrent or newly diagnosed glioblastoma, including XL765 a dual PI3K/mTOR inhibitor and BKM-120, an oral PI3K inhibitor.

PDGFR inhibitors

PDGFR signaling is also important for glioma progression, and several inhibitors of this pathway are currently under testing. Imatinib, a small molecule which inhibits Bcr-Abl, c-Kit and PDGFR kinases have shown minimal benefit (Wen, Yung et al. 2006). Second-generation of PDGFR inhibitors with improved central nervous system penetration such as

tandutinib or dasatinib are currently undergoing phase I and II clinical trials.

CDKs inhibitors

Due to the high frequency of mutations in Rb signaling pathway in glioma patients, there are novel drugs targeting this pathway. PD-0332991, an inhibitor of CDK4 and CDK6 is currently under phase II clinical trials for recurrent glioblastoma with known Rb-pathway alterations. Preclinical studies suggested that this may be effective in reducing glioblastoma growth (Michaud, Solomon et al. 2010).

Histone Deacetylases inhibitors

Another approach for targeting glioma is the inhibition of histone deacetylases (HDACs), regulators of chromatin structure and gene expression which are frequently mutated or altered in GBM. LBH589 and Vorinostat are now being tested in phase II clinical trials for recurrent glioblastoma (Galanis, Jaeckle et al. 2009). Notably, valproic acid, an antiepileptic agent with HDAC inhibitory effect, has been associated with survival benefit in glioblastoma patients when administrated in combination with temozolamide and radiation therapy (Weller, Gorlia et al. 2011). A phase II clinical trial is being conducted using valproic acid in newly diagnosed glioblastoma patients.

Antiangiogenic therapies

Angiogenesis is one of the main features of GBM and VEGF is a key mediator of angiogenesis in glioblastoma. Bevacizumab (Avastin) has been approved by the FDA as a monotherapy for recurrent glioblastoma in 2009 based in radiographic responses. Treatment with Bevacizumab resulted in an increase of 29-46% in 6-month progression-free survival rates (Cohen, Shen et al. 2009; Friedman, Prados et al. 2009). Bevacizumab has been

investigated in combination with other targeted therapies such as irinotecan, erlotinib, or with radio or chemotherapy.

There are other anti-VEGF therapies such as aflibercept or cediranib. Sorafenib, which also inhibits other RTKs (PDGFRβ, BRAF, c-Kit and Raf) has been tested as monotherapy or in combination with no promising results.

Cilengitide is a selective inhibitor of αvβ3 and αvβ5 integrins, adhesion molecules that facilitate endothelial proliferation and migration (Reardon, Fink et al. 2008). It was in clinical trials but it did not show any promising results.

Other drug aimed to inhibit pro-angiogenic pathways is AMG386, which sequesters angiopoietin 1 and 2, and is being tested as a single agent and in combination with bevacizumab.

Immunotherapies

Malignant gliomas are associated with immunesupression. Several preclinical studies showed promising results in vaccination strategies. CDX-110 is an EGFRvIII peptide vaccine which is the most advanced experimental immunotherapy for glioblastoma patients. There are also vaccines composed of heat shock proteins (HSPs) conjugate with tumor antigens, which are injected sub-cutaneous into patients.

Agent	Target	Selected reference/trial identifier
EGFR-targeted therapies		
Erlotinib	EGFR	Brown *et al.* (2008),[57] van den Bent *et al.* (2009)[60]
Gefitinib	EGFR	Franceschi *et al.* (2007),[173] Kreisl *et al.* (2009)[69]
Cetuximab	EGFR	Neyns *et al.* (2009)[62]
Lapatinib	EGFR, HER2	Thiessen *et al.* (2010)[63]
Afatinib	EGFR	NCT00977431
Dacomitinib	EGFR	NCT01112527, NCT01520870
Nimotuzumab	EGFR	NCT00753246
PI3K/Akt/mTOR-targeted therapies		
Temsirolimus	mTOR	Galanis *et al.* (2005)[68]
Everolimus	mTOR	Kreisl *et al.* (2009)[69]
BKM120	PI3K	NCT01339052, NCT01349660, NCT01473901
XL765	PI3K, mTOR	NCT00704080
Others		
Sorafenib	PDGFR-α,β, VEGFR-2,3, BRAF, c-Kit, Ras	Hainsworth *et al.* (2010),[108] Reardon *et al.* (2011),[109] NCT01434602, NCT00329719, NCT00734526, NCT00884416
Imatinib	PDGFR-α,β, c-Kit, Bcr–Abl	Wen *et al.* (2006),[74] Reardon *et al.* (2009),[75] Dresemann *et al.* (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 *et al.* (2009),[81] Friday *et al.* (2012)[82]
Panobinostat	Histone deacetylase	NCT00859222
Valproic acid	Histone deacetylase	NCT00302159
Bortezomib	Proteasome	Friday *et al.* (2012),[82] Phuphanich *et al.* (2010)[83]
Iniparib	Poly(ADP-ribose) polymerase	NCT00687765
GSC-targeted therapies		
R04929097	γ-secretase	NCT01119599, NCT01122901
Vismodegib	Smoothened homolog	NCT00980343
Antiangiogenic therapies		
Bevacizumab	VEGF-A	Friedman *et al.* (2009),[96] Lai *et al.* (2011)[98]
Aflibercept	VEGF-A, VEGF-B, PlGF	de Groot *et al.* (2011)[102]
Cediranib	VEGFR-1,2,3, PDGFR-α,β, FGFR-1, c-Kit	Batchelor *et al.* (2007)[103]
Sorafenib	VEGFR-2,3, PDGFR-α,β, BRAF, c-Kit, Ras	Hainsworth *et al.* (2010),[109] Reardon *et al.* (2011)[110]
Sunitinib	VEGFR-2, PDGFR-β, c-Kit, RET, Flt3	Neyns *et al.* (2011)[116]
Vandetanib	VEGFR-2, EGFR, RET	Drappatz *et al.* (2010)[117]
Cabozantinib	VEGFR-2, Met, RET, c-Kit, Flt3, Tie-2	Wen *et al.* (2010)[115]
Cilengitide	αvβ3,αvβ5 integrins	Reardon *et al.* (2008),[121] Stupp *et al.* (2010)[122]
Enzastaurin	PKC-β, Akt	Kreisl *et al.* (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.

Figure 1.9. Targeted therapies in glioblastoma. List of the different compounds that are being tested for GBM treatment, and their targets. From (Tanaka, Louis et al. 2013).

GLIOMA MOUSE MODELS

Animal models of glioma are important tools, not only to study the biology of the disease and improve the understanding of gliomagenesis, but also for preclinical studies to develop new therapeutic approaches. *In vitro* experiments with cell lines or patient-derived cells have the inherent limitation that there is no interaction with tumor stroma, tumor microenvironment and angiogenesis. Thus, there is a need to develop reliable and near-to-clinic glioma mouse models (Holland 2001; Holland 2001; Wee, Charles et al. 2011).

There are two main *in vivo* models for glioma:

a. Genetically engineered mouse models (GEMM)

Originally, these models were achieved by treating animals with mutagenic agents (Donehower, French et al. 2005). However, such tumors are induced by spontaneous mutations and do not resemble the stages in actual patient tumors. In order to study the role of certain mutations described in glioma, and taking advantage of the recent advances in genetic engineering, there are many genetically engineered mouse models (GEMM) of glioma. One of the first models of GBM was generated using RCAS viral system, where RCAS virus derived from avian sarcoma is used to deliver the expression of an oncogene, in this case K-RAS or PDGFB (Holland 2001; Hambardzumyan, Amankulor et al. 2009).

Mouse models for pro-neural (*PDGFA* amplification-driven) and mesenchymal subtypes (*NF1* loss-driven) have been recently developed (Hambardzumyan, Cheng et al. 2011). There are also

transgenic mouse models driven by *EGFR* amplification or mutation (EGFRvIII) in combination with *PTEN* or *CDKN2A* loss that recapitulate the classical phenotype found in GBM patients (Altshuler, Tekell et al. 2007).

b. Implantation of tumor cells

When the implanted cells are originally from the same animal or same species it is considered an allograft. If cells are from different species it is called a xenograft. It is very common to use human patient-derived cells or human immortalized cell lines to generate tumors in mice.

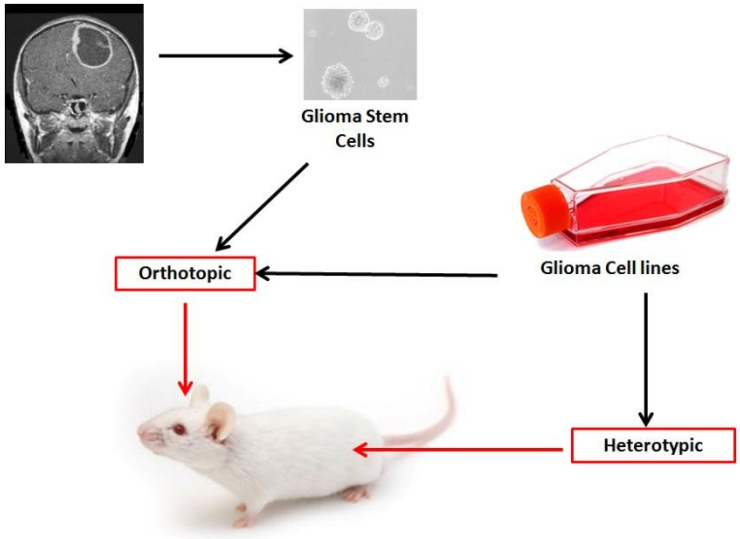


Figure 1.10. Different mouse models of tumor cell implantation. Glioma cell lines or glioma stem cells derived from patients are inoculated into immunocompromized mice. In an **orthotopic** model cells are inoculated into the same tumor site, in this case, the brain. A **heterotypic** model is when cells are inoculated in a different site, usually subcutaneously, in the mouse flank.

In this case, immunocompromised mice are used to avoide immune system rejection of implanted cells. The moste frequently used mice are Athymic Nude-Foxn1nu, Non-Obese Diabetic NOD.CB17-Prkdc^{scid} or NOD *scid* gamma NOD.Cg-Prkd^{scid} Il2rg^{tm1wjl}/SzJ (NSG). Depending on the site of implantation orthotopic models are used (if cells are implanted in the same site of the original tumor) or heterotypic model (if cells are implanted in a different location than the original tumor). In the case of glioma, in an orthotopic model we implant the cells in the brain, while in a heterotypic model we implant them subcutaneously (Figure 1.10) (Morton and Houghton 2007; Talmadge, Singh et al. 2007).

The model used in this project is an orthotropic xenograft model using patient-derived cells isolated from GBM. This model can be used to study GICs that are isolated from patients and thus have the same characteristics at level of mutations, gene expression and genomic alterations (Figure 1.11) (Anido, Saez-Borderias et al. 2010; Wee, Charles et al. 2011). In our case, as we are interested in pre-clinical studies using compounds assessed in the clinics, a patient-derived xenograft will better predict the response to certain drugs, although we are obviously missing all the immune system effect which can be critical (Richmond and Su 2008).

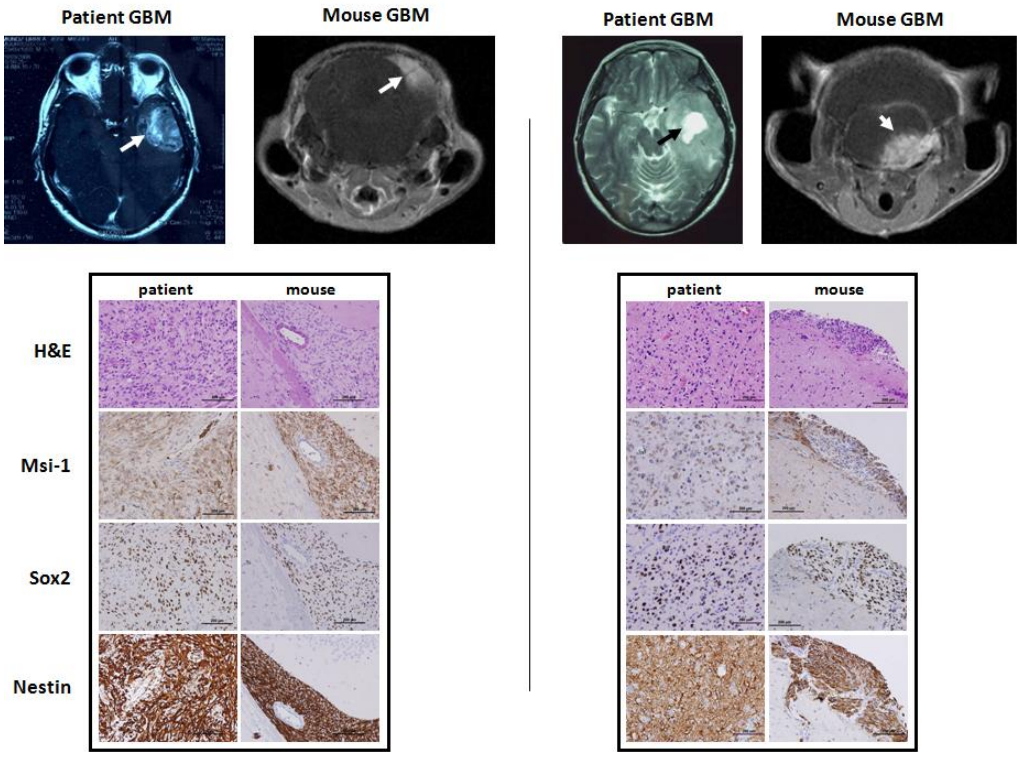


Figure 1.11. Our glioblastoma xenograft mouse model recapitulates the characteristics from patient's tumor. Comparison between patient and patient-derived mouse model for two different GBM samples. Mouse tumors are very similar to patient's in terms of localization, histology and expression of different markers. Adapted from (Anido, Saez-Borderias et al. 2010).

2. GLIOMA INITIATING CELLS (GICs)

In addition to different tumor subtypes, cells within the tumor bulk often exhibit functional heterogeneity, harboring distinct capacities (Heppner and Miller 1983; Visvader and Lindeman 2008). As discussed earlier in this chapter, GBM is a very heterogeneous tumor. In the previous chapters we have described the **inter-tumoral heterogeneity**, which means that different patients with the same diagnosis will have a singular tumor with differences in gene expression, genomic aberrations and cellular composition, thus leading to significant differences that may be taken into account at the time of therapeutic decision. But there is also an important type of heterogeneity within the same patient: the **intra-tumoral heterogeneity**. Not all the cells within the same tumor bulk necessarily share the same characteristics. Intra-tumoral heterogeneity is very important as different cellular populations might determine the response to treatment and would lead to treatment resistance.

Two models have been proposed to explain tumor initiation and cellular heterogeneity found in GBM. First, the **stochastic model** (Figure 1.12A), which postulates that each cell within the tumor is equally malignant and has the capacity to initiate and maintain the tumor through constant duplication. The heterogeneity and different properties of the cells are attributed to genomic instability caused by initial oncogenic mutations and different interactions with the tumor microenvironment. Recently, another model has been proposed, the **cancer stem cell** model or **hierarchical model** (Figure 1.12B). This hypothesizes that a defined subset of tumor cells, called cancer stem cells or **cancer**

initiating cells (CICs), have the capacity to initiate tumor growth, maintain proliferation and generate recurrent tumors.

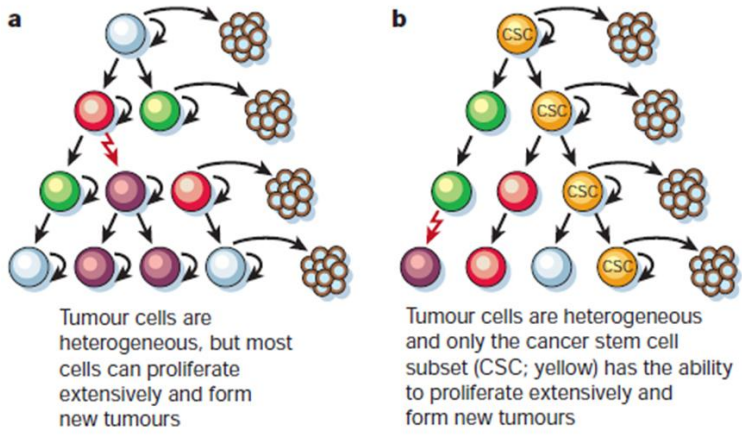


Figure 1.12. Two proposed models for cancer evolution. The **stochastic model (a)** and the **hierarchical model (b)**. In the first model, all cells within a population can receive the oncogenic hit and generate a progeny of cells with the same characteristics. In the second, cells with stem-cell characteristics are the ones that receive the oncogenic hit and are able initiate the tumor and generate all the different cell types found in the tumor. From (Reya, Morrison et al. 2001).

These cancer initiating cells generate heterogeneous cell populations that comprise the tumor and maintain themselves through self-renewing divisions but simultaneously give rise to progenitor cells. In glioma, several authors have demonstrated their existence and characterized Glioma Initiating Cells which share some stem cell characteristics (Reya, Morrison et al. 2001; Fomchenko and Holland 2005; Sanai, Alvarez-Buylla et al. 2005; Chen, Chinnaswamy et al. 2007; Kim and Dirks 2008; Sulman, Aldape et al. 2008; Piccirillo, Combi et al. 2009; Woolard and Fine 2009; Natsume, Kinjo et al. 2011). In the adult human brain, the neural stem cell compartment is located in the subventricular zone of the lateral ventricles and near the dentate gyrus ependyma in the temporal horn (Sanai, Alvarez-Buylla et al.

2005; Alvarez-Buylla, Kohwi et al. 2008; Jackson and Alvarez-Buylla 2008). This population is defined by its self-renewing (symmetric cell division) capacity and ability to give rise to different lineage-committed cells with neuronal, astrocytic or oligodendrocytic characteristics (asymmetric cell division). These properties can be regulated by intrinsic factors or by interactions with their microenvironment (Dirks 2008; Dirks 2008). Several similarities can be found between tumor stem cells and Glioma Initiating Cells (GICs). GICs are also capable of **self-renewing** and give rise to **distinct populations** within the tumor (Dirks 2008).

Another important feature of GICs is the **resistance to conventional therapies** such as radio and chemotherapy (Barker, Simmons et al. 2001; Bao, Wu et al. 2006; Rich 2007; Sheehan, Shaffrey et al. 2010). This is a critical issue in the case of glioma, since one of the major causes of death in glioma is tumor recurrence. Recent findings point out that Nestin-expressing neural stem cells may be the cell of origin in the case of glioma and are able to reconstitute a tumor after irradiation (Chen, Li et al. 2012).

Brain tumor stem cells were first isolated by their ability to grow in non-adherent conditions in serum-free media supplemented with EGF and basic Fibroblast Growth Factor (bFGF) (Reynolds and Weiss 1992; Vescovi, Reynolds et al. 1993). GICs grown as neurospheres show a sustained self-renewal capacity and proliferation (Singh, Clarke et al. 2003) and can generate a progeny that is able to differentiate into astrocytes, oligodendrocytes and neurons (Galli, Binda et al. 2004).

Although there is some controversy concerning the nature of these cells, it is clear that at least some of the typical stem-cell signaling pathways are operative in GICs. For example **Notch**, **Sonic Hedgehog (Shh)** and **Wnt** pathways seem to be important for the proliferation and survival of GICs

(Reya, Morrison et al. 2001; Fan, Khaki et al. 2010; Natsume, Kinjo et al. 2011). In addition, the **TGFβ** (Penuelas, Anido et al. 2009; Seoane 2009), and **MET** pathways have been demonstrated to be crucial for the maintenance of GIC properties (Watabe and Miyazono 2009; Joo, Jin et al. 2012).

There have been several attempts to define the GIC population. In order to isolate and characterize GICs, there is a need of defining markers of this population. The usage of different markers varies depending on the authors and model of study. Although some studies postulate that GICs are characterized by expression of CD133 surface protein (Singh, Clarke et al. 2003; Tso, Shintaku et al. 2006; Beier, Hau et al. 2007), this is unclear, as there are also CD133 negative cells having cancer initiating capacity (Rao, Vivekchand et al. 2007; Son, Woolard et al. 2009; Chen, Nishimura et al. 2010; Ma, Ma et al. 2013). Other authors postulate that GICs can be identified by the expression of ABC transporter or by Hoechst 33342 dye exclusion by FACS-flow cytometry, defined as a Side Population (Buijs, van der Horst et al. 2012). There is an imperious need to improve identification and characterization of this population, as they are responsible for cancer initiation, tumor resistance and relapse after treatment.

TARGETING GLIOMA INITATING CELLS

Recent evidences showing the importance of GICs, especially in conferring resistance and driving the relapse after treatment, suggested this entity might be an attractive target for new treatments. Drugs that target GICs in combination with radiotherapy or chemotherapy might prevent recurrence

(Bao, Wu et al. 2006). Notch pathway and Sonic-Hedgehog (Shh) pathways are critical for GICs. RO4929097 is an inhibitor of ɣ-secretase which blocks Notch pathway activation and is being evaluated in phase II clinical trials for patients with recurrent glioblastoma. Vismodegim and oral small inhibitor of Shh signaling is going to be assessed in GBM patients.

GICs TEND TO BE LOCATED IN A PERIVASCULAR NICHE IN GLIOBLASTOMA

Several reports show the importance of the relationship between tumor cells and surrounding microenvironment, which has a crucial role in contributing to tumor initiation, progression and metastatic capacity of cancer cells (Hu and Polyak 2008; Polyak, Haviv et al. 2009; Barcellos-Hoff, Lyden et al. 2013).

Cancer Initiating cells, as well as normal embryonic stem-cells, tend to stay at particular locations or niches, and depend on the local microenvironment (Spradling, Drummond-Barbosa et al. 2001; Ohlstein, Kai et al. 2004; Moore and Lemischka 2006; Borovski, De Sousa et al. 2011; Medema and Vermeulen 2011; Shestopalov and Zon 2012; Takakura 2012). Niches are composed by non-tumor cells (inflammatory cells, endothelial cells, fibroblasts…) and the extracellular matrix (ECM). Those provide direct cell contacts, interactions and secrete factors that maintain stem cells in a quiescent state, regulating their self-renewal capacity and multipotency. Diverse genetic and molecular analyses have identified many factors and cytokines that support stem-cell niches, including components of Notch, Wnt, and Sonic hedgehog (Shh) signaling pathways (Visvader and Lindeman 2008; Wang, Li et al. 2009). As examples of well studied stem-

cell niches, Intestinal Stem Cells, characterized by the expression of Lgr5 marker, reside in a niche at the bottom of intestinal crypts in association with Paneth cells (Barker, van Es et al. 2007) and Hair-Folicle Stem Cells (HFSCs) are located in the bulge, located below the sebaceous glands of the hair follicles where the levels of different cytokines regulate the transitions between quiescent and activated state (Tumbar, Guasch et al. 2004). In this case, the balance between BMPs and TGF-β regulates the HFSCs activation cycle (Oshimori and Fuchs 2012).

In the case of CICs, several authors reported their presence in specific niches. In the case of cancer, the recruitment of inflammatory cells, endothelial cells and myofibroblasts leads to the stablishment of a complex network of growth factors, chemoquines, hormones, enzymes and ECM that promote the CIC traits (Joyce and Pollard 2009; Cabarcas, Mathews et al. 2011; Korkaya, Liu et al. 2011).

In different types of tumors, CICs are found to be located near stromal cells, suggesting an intimate collaboration between CIC and tumor microenvironment.

CICs are not passively residing in the niche, but they can also interact and modify the niches trough a complex crosstalk between different components of the tumor microenvironment as shown in Figure 1.13. As an example, GICs secrete VEGF to promote tumor angiogenesis and this is correlated with an increased tumor-initiating capacity (Bao, Wu et al. 2006). Either the niche can affect stem cells and their properties, or stem-cells are capable to influence on their microenvironment, creating a close relationship between stem cells and their niche.

Recent evidences show that CICs can also recruit different immune cells, modulating their normal functioning and promoting tumor inflammation, which in turn, support the maintenance of the CIC pool (Filatova, Acker et al. 2013). Interestingly, TGF-β has a well described role maintaining CICs in different tumor types as well as modulating immune response, suggesting that TGF-β could have an important role in CIC niches.

TGF-β has a relevant role in tumor microenvironment, mediating the interactions between cancer cells and their niche. TGF-β can be secreted by both, tumor cells or stroma/microenvironment cells in a finely regulated balance (Stover, Bierie et al. 2007). TGF-β has an important autonomous autocrine and paracrine effect over cancer cells, but it also can be modulated by several factors in the tumor microenvironment, such as fibroblasts, immune cells and ECM (Bierie and Moses 2006). The complex interaction between TGF-β, CICs and cells from the niche is a subject that needs to be further studied.

In the case of neural stem cells, they are located in the proximity of ventricles, close to ependymal cells in the Subventricular Zone and also near to blood vessels. They need to be in their specific niche to maintain their undifferentiated state and self-renewal capacity (Gust, Biswas et al. 2007; Tavazoie, Van der Veken et al. 2008; Charles, Holland et al. 2011). In the case of GICs, it has been postulated that they also reside in specific niches, where there are certain GFs and cytokines that maintain their stem cell capacity (Heddleston, Hitomi et al. 2011; Lathia, Heddleston et al. 2011). It has been described that GICs reside in two different and specific niches: the **perivascular niche**, near the tumor blood vessels, and the **hypoxic niche**, distant to blood vessels and where oxygen and nutrients are scarce and there is activation of HIF1α transcription factor (Gilbertson

and Rich 2007; Lathia, Heddleston et al. 2011). At the same time, tumor endothelial cells may derive from tumor cells, suggesting that GIC are capable to create their own niche (Ricci-Vitiani, Pallini et al. 2010; Wang, Chadalavada et al. 2010).

In the case of perivascular niche, GICs located in the proximity of tumor vessels interact with endothelial cells which support GICs and provide GFs and cytokines necessary to maintain their undifferentiated state (Gilbertson and Rich 2007; Oka, Soeda et al. 2007; Galan-Moya, Le Guelte et al. 2011; Zhu, Costello et al. 2011). At the same time, tumor cells secrete many pro-angiogenic factors to support and promote angiogenesis and endothelial cell proliferation (Figure 1.14) (Dunn, Heese et al. 2000; Gilbertson and Rich 2007). Furthermore, tumor endothelial cells may derive from tumor cells, suggesting that GIC are capable to create their own niche (Ricci-Vitiani, Pallini et al. 2010; Wang, Chadalavada et al. 2010).

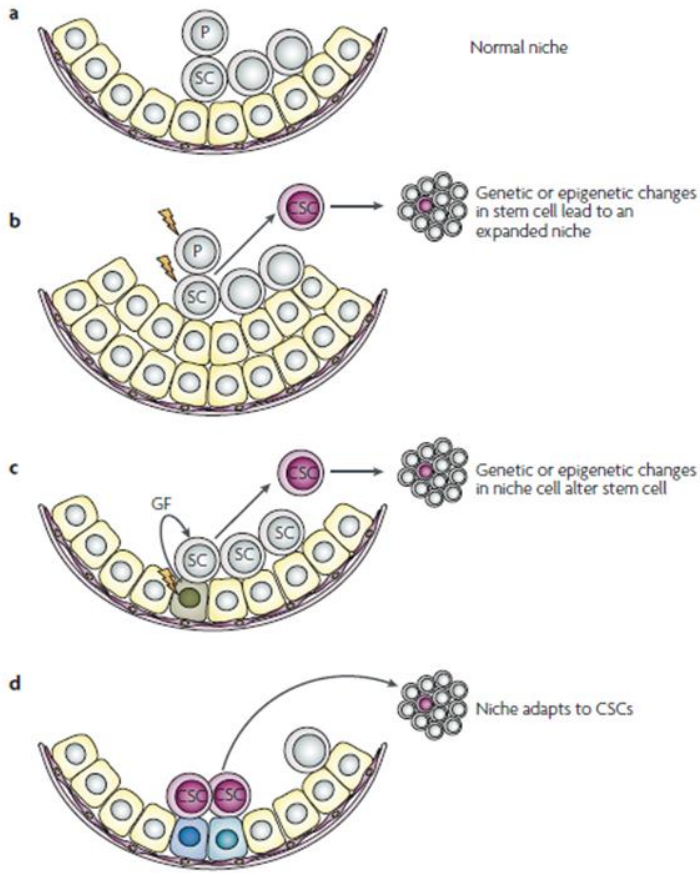


Figure 1.13. Stem cells and their interactions with the niche. **A.** Normal stem cells are located in their niches from where they receive growth factors and a microenvironment that maintains them in their undifferentiated state. **B**. Oncogenic alterations in stem cells lead to tumor stem cells which can modify the niche and cause its expansion. **C.** Alterations in cells from the niche can increase GF release and cause hyperprolifearion of stem cells. **D.** Cancer stem cells have the capacity to modify their niche in order to sustain their needs. From (Buijs, van der Horst et al. 2012).

Some authors propose that targeting this perivascular niche may be effective in order to eradicate the GICs population, thus decreasing the probability of tumor relapse (Folkins, Man et al. 2007; Yang and Wechsler-Reya 2007).

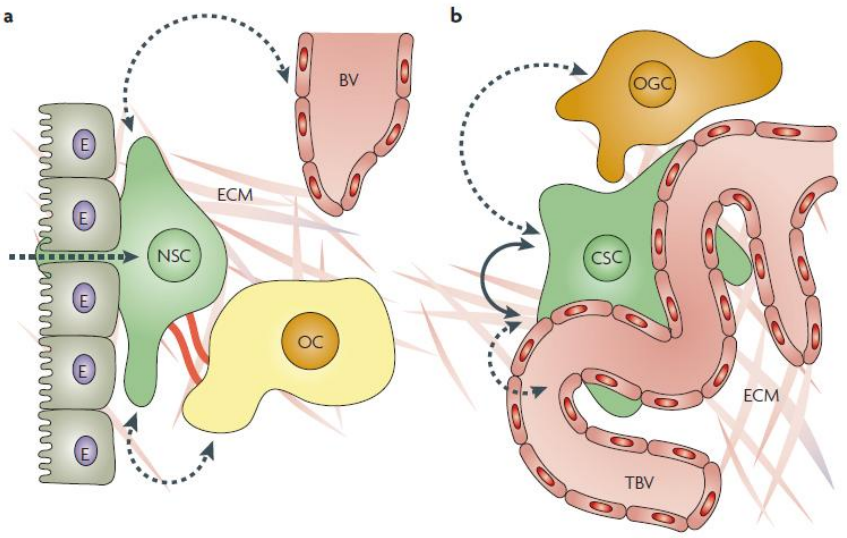


Figure 1.14. Glioma initiating cells tend to be located in a perivascular niche. Similarly to normal neural stem cells, which are located in the proximity of blood vessels and ependymal cells (**a**), glioma initiating cells tend to be located in the proximity of tumor blood vessels (**b**). From (Gilbertson and Rich 2007). Abreviations: B: Blood vessel, NSC: Neural Stem Cell, ECM: Extracellular Matrix, E: Ependymal cell, OC: Other Cell, CSC: Cancer Stem Cell, TBV: Tumor Blood Vessel, OGC: Other Glioma Cell

3. THE TRANSFORMING GROWTH FACTOR BETA (TGFB) PATHWAY

Transforming Growth Factor β (TGFβ) was first isolated and characterized in 1984 (Massague 1984; Massague 1985; Massague and Like 1985).

TGFβ belongs to the TGFβ super family which is composed by TGFβ (TGFβ 1, 2 and 3), Bone Morphogenic Proteins BMPs (BMP 2 to 15), Activin, Nodal and Anti-Müllerian Hormone (AMH).

TGFβ is a cytokine that maintains normal tissue homeostasis and it is a key regulator of stem cell differentiation during embryonic development (Massague, Blain et al. 2000; Massague 2012; Massague 2012). It signals through a Serine-Threonine kinase heterodimeric receptor formed by the type I (TβRI) also known as ALK, and type II (TβRII) receptor (Wrana, Attisano et al. 1992; Massague 1996; Massague 2000; Massague and Chen 2000).

Molecular category	TGFβ pathway*	Activin/inhibin/Nodal pathway*	BMP pathway*
Ligands	TGFβ1, TGFβ2, TGFβ3	Activin A, activin B, inhibin A, inhibin B, Nodal	BMP2, BMP4, BMP5, BMP6, BMP7, BMP8A, BMP8B, BMP9, BMP10
Type I receptors	TβRI (ALK5), ALK1 (ACVRL1 or SKR3)	ALK4 (ACVR1B or ACTRIB), ALK7 (ACVR1C or ACTRIC)	ALK1 (ACVRL1, SKR3), ALK2 (ACVR1, ACTRI), ALK3 (BMPR1A), ALK6 (BMPR1B)
Type II receptors	TβRII	ACTRIIA, ACTRIIB	BMPR2, ACTRIIA, ACTRIIB
Type III receptors	TβRIII (betaglycan), endoglin, CRIPTO3 (TDGF1P3)	CRIPTO1 (TDGF1), CRIPTO3 (TDGF1P3), TβRIII (betaglycan)	RGMA, RGMB (DRAGON), RGMC (HJV or HFE2), endoglin
R-SMADs	SMAD2, SMAD3	SMAD2, SMAD3	SMAD1, SMAD5, SMAD8
Co-SMAD	SMAD4	SMAD4	SMAD4
I-SMADs	SMAD7	SMAD7	SMAD6, SMAD7

*Alternative protein names are listed in brackets. ACTR, activin receptor; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; BMPR, BMP receptor; RGM, repulsive guidance molecule; TβR, TGFβ receptor; TDGF, teratocarcinoma-derived growth factor.

Figure 1.15. Different ligands and receptor combinations of the TGFβ superfamily members. Depending on the TGFβ family member, the receptors and Smads that are activated vary. From (Akhurst and Hata 2012).

Upon ligand binding of TGFβ dimers to TβRII, there is recruitment and phosphorylation of TβRI by TβRII. TβRI in turn, phosphorylates Receptor-activated Smad 2 and 3 (R-Smads) in its carboxy terminal SXS motif, releasing them from the cytoplasm and allowing them to bind Smad4 and translocate to the nucleus to regulate transcription (Massague and Chen 2000; Massague, Seoane et al. 2005; Schmierer and Hill 2007; Massague 2008; Ikushima and Miyazono 2010; Massague 2012) (Figure 1.15).

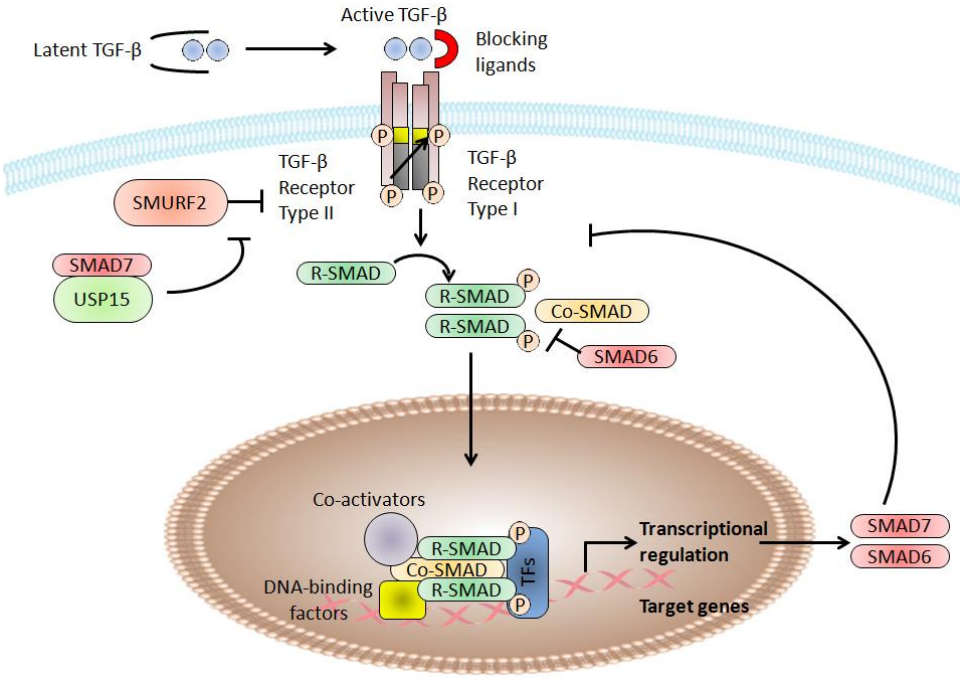


Figure 1.16. Schematic representation of the TGFβ pathway. TGFβ activates its receptor type I and II which phosphorylates and activates Smads. Once phosphorylated Smads form complexes that are shuttled into the nucleus and bind to other Transcription Factors (TFs) and co-activators or co-repressors to orchestrate the transcriptional program. TGFβ pathway activity is down-modulated by Smad6 and Smad7 and poly-ubiquitination of the TypeI receptor by SMURF2. USP15 counteracts this poly-ubiquitination and up-regulates pathway activity.

TGFβ is a pleiotropic cytokine which triggers a wide variety of gene responses depending on the cellular context. These diverse responses are regulated by the binding of Smads to other transcription factors that act as cofactors (Shi and Massague 2003; Massague, Seoane et al. 2005). Smad transcription factor MH1 domain recognizes CAGA sequences and certain GC-rich sequences. However, the affinity of Smads for DNA is very low, requiring the cooperation of other transcription factors. Those cofactors may act as activators or repressors of gene expression, determining the different responses to TGFβ-pathway activation (Massague 1996; Zawel, Dai et al. 1998; Massague, Seoane et al. 2005).

Besides the canonical Smad signaling pathway, TGFβ can also trigger other important signaling pathways, such as PI3K and MAPK pathways, which are crucial for many of the TGFβ effects (Miyazono 2009).

TGF-β pathway is physiologically tightly regulated at many different levels. First of all, every TGF-β isoforms is synthesized as a precursor, which forms a homodimer that interacts with Latency-associated protein (LAP) and latent TGF-β-binding protein (LTBP). Cleavage of this complex is necessary to release active TGF-β that can bind to its receptors. Matrix Metallo-Proteases 2 and 9 (MMP2 and 9) and Thrmobospondin-1 (THBS1) are involved in the activation of latent TGF-β (Shi, Zhu et al. 2011). Also, the interaction of the ligands with the receptor can be blocked by extracellular antagonists. For example Activins can be blocked by binding to Follistatin, Lefty blocks and inhibits Nodal signalling and BMP ligands are blocked by the inhibitor Coco (Massague and Chen 2000; Massague and Gomis 2006). Another level of regulation occurs through inhibitory Smads (Smad6 and

Smad7) and Skil, which decrease the activity of the pathway (Massague, Seoane et al. 2005; Moustakas and Heldin 2009). Smad7 binds to the type I Receptor, preventing the phosphorilation of R-Smads and Smad6 binds to the co-Smad (Smad4) preventing the nuclear transport of R-Smads (Massague and Chen 2000). Skil (also known as SnoN) bind to R-Smad and Smad4 complexes, disrupting the complexes and competing with other cofactors that are needed for the signaling activity (Deheuninck and Luo 2009). They are downstream targets of the TGF-β pathway, creating a negative feedback loop tightly controlling the pathway activity. TGF-β signaling can also be attenuated by polyubiquitylation and proteasome-mediated degradation. The E3 ubiquitin-protein ligases SMURF1, SMURF2 and NEDD4L target Smads and TGF-β receptors for degradation (Wrana, Attisano et al. 1994; Kavsak, Rasmussen et al. 2000; Wicks, Grocott et al. 2006; Itoh and ten Dijke 2007). USP15 has been recently described to counteract this, de-ubiquitinating RSmads and TβRI, thus increasing the TGF-β signaling in a fine regulated manner (Inui, Manfrin et al. 2011; Eichhorn, Rodon et al. 2012).

THE TGFβ PATHWAY IN CANCER

In the following pages we are going to revise the most well-known and studied properties of TGFβ as an oncogenic factor. TGFβ has an important role promoting tumorigenesis and metastasis and it has been studied for decades.

TGFβ typically acts as a potent **inhibitor of the cell cycle** (tumor suppressor) in normal epithelial cells or astrocytes. In cancer, there are many alterations in the TGFβ pathway such as mutations in the TGFβ

Receptor (in ovarian, head and neck, colon and gastric cancers), mutations in Smads (in pancreatic cancer) and alterations or mutations in different cofactors (as found in breast cancer or glioblastoma) (Massague 2008). For example FoxO (Forkhead class O) transcription factor is the cofactor for Smads in the induction of $p21^{Cip1}$. In GBM, hyperactivation of the PI3K pathway negatively regulates FoxO factors, while the high levels of FoxG1 found in some GBM patients may inhibit the activity of FoxO as Smad partners; both changes prevent $p21^{Cip1}$ induction by TGFβ (Seoane 2004). These alterations in the TGFβ signaling pathway inhibit the cytostatic program of TGFβ.

In some advanced tumors, among them high-grade glioma, TGFβ can act as an **oncogene** in contrast with its anti-proliferative role. In these cases TGFβ promotes tumor cell proliferation, invasion, metastasis and angiogenesis (Akhurst 2004; Bruna, Darken et al. 2007; Massague 2008; Seoane 2008). Secreted TGFβ affects not only tumor cells but also stromal cells, where it promotes the production of pro-tumorigenic cytokines, modulates the microenvironment and suppresses the immune system, allowing for tumor escape (Bierie and Moses 2006; Massague 2008).

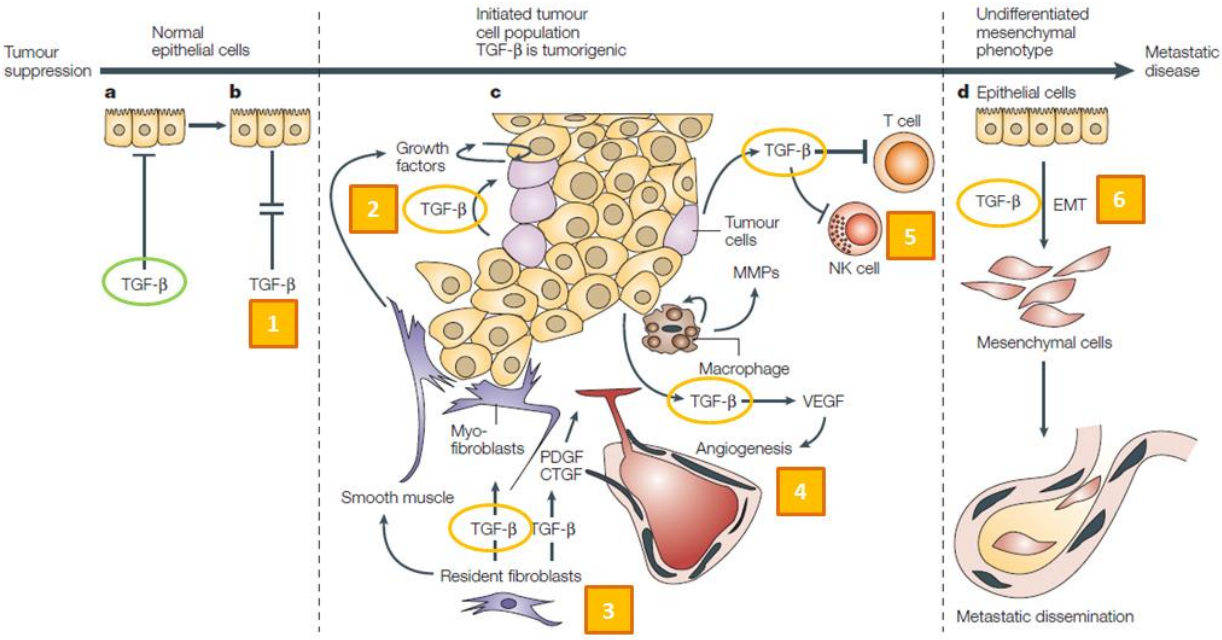


Figure 1.17. Diverse roles of TGFβ in tumor progression. In normal epithelial cells TGFβ has a tumor suppressor role inhibiting cell cycle and inducing apoptosis. In early carcinogenesis, cells evade the TGFβ anti-proliferative effect (**1**). In advanced cancers, TGFβ has an important oncogenic role promoting cell proliferation and secretion of other growth factors (**2**). There is also an important secretion of TGFβ by other cells from the tumor stroma, creating a favorable microenvironment for tumor growth (**3**). TGFβ also promotes angiogenesis through the induction of VEGF (**4**) and is a potent immunosuppressor inhibiting the immune response against the tumor (**5**). In metastatic disease, TGFβ promotes EMT, inducing the metastatic dissemination of tumor cells (**6**). Adapted from (Yingling, Blanchard et al. 2004).

TGFβ INDUCES EMT AND PROMOTES METASTASIS

Another important and well-characterized role of TGFβ oncogenic effect is in the **Epithelial to Mesenchymal Transition** (EMT) (Heldin, Vanlandewijck et al. 2012; Massague 2012). EMT is a well-coordinated process that occurs during embryonic development. It is characterized by the loss of E-Cadherin and other components of epithelial cell junctions and the acquisition of a more motile mesenchymal phenotype. Upon EMT, apical-basal cell polarity is lost and cells acquire a spindle-shaped morphology and express mesenchymal markers such as N-Cadherin, Vimentin, Fibronectin, Smooth Muscle Actin (SMA) and Fibroblast-Specific Protein-1 (FSP-1)(Heldin, Vanlandewijck et al. 2012). The resulting mesenchymal cells secrete extracellular matrix proteases (MMPs) and have increased motility and invasive properties (Miyazono 2009; Heldin, Vanlandewijck et al. 2012). EMT is a key process that occurs during gastrulation and formation of neural crest, somites, heart and craniofacial structures, typically driven by a set of transcription factors including Snail, Slug, Twist, ZEB-1 and 2 and FoxC3 (Massague 2008). EMT is also an important step in the invasion and metastasis of cancer (Heldin, Vanlandewijck et al. 2012; Miyazono, Ehata et al. 2012). EMT contributes to tumor invasion and dissemination due to the motile phenotype that it confers upon tumor cells. TGFβ is a very potent inducer of EMT, inducing the expression of several transcription factors involved in EMT including ZEB1 and 2, Snail and Slug (Massague and Wotton 2000; Miyazono 2009).

TGFβ also promotes distal metastasis. Approximately 40% of patients with breast metastasis show a TGFβ response signature with high expression of *TGFβ1*, *TGFβ2*, *LTBP1*, *SMAD3* and *SMAD4*. This TGFβ

gene response signature status was associated with those patients harboring lung metastasis (Padua and Massague 2009). One of the key mediators of TGFβ metastatic effect is the induction of angiopoietin-like 4 (ANGPTL4). TGFβ induces *ANGPTL4* in the primary tumor and this is important for tumor extravasation as Angptl4 disrupts vascular endothelial cell to cell junctions, facilitating distant metastasis seeding (Padua, Zhang et al. 2008). Once metastasis is seeded, TGFβ also has an important role in promoting tumor reinitiation in the case of bone or lung metastasis (Massague 2008; Padua and Massague 2009). As an example, in breast cancer cells that have entered to lung parenchyma, TGFβ facilitate tumor reinitiation through an aberrant induction of *ID1* expression (Padua, Zhang et al. 2008).

TGFβ INDUCES TUMOR ANGIOGENESIS

TGFβ signaling also induces angiogenesis in some tumors. Tumor angiogenesis is essential for tumor growth and metastasis. TGFβ functions as a pro-angiogenic factor *in vivo*. Increased expression of TGFβ is correlated with higher vascular density in some tumors. TGFβ is able to induce expression of Connective Tissue Growth Factor (CTGF) and Vascular Endothelial Growth Factor (VEGF), as well as increasing the synthesis of Matrix Metallo-Proteases (MMPs), which lead to stimulation of migration and invasion of vascular endothelial cells, resulting in accelerated tumor angiogenesis (Bertolino, Deckers et al. 2005; Miyazono, Ehata et al. 2012).

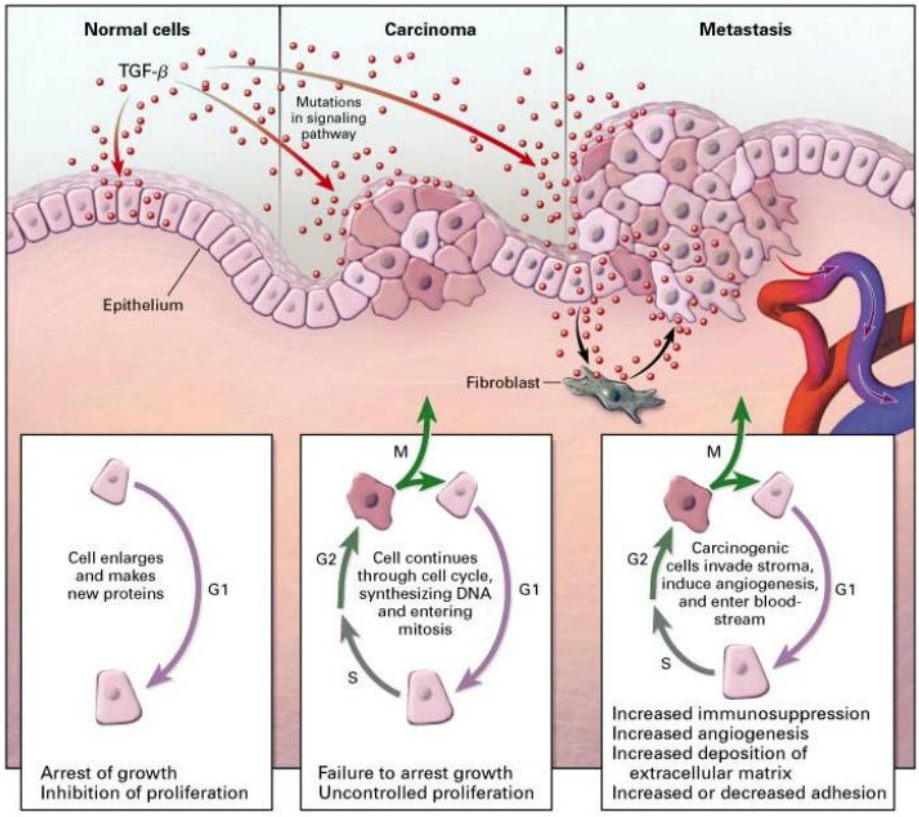


Figure 1.18. The TGFβ pathway and its role in cancer. TGFβ has a tumor suppressor role in normal epithelial cells and in early stages of tumor progression promoting cell cycle arrest, differentiation and apoptosis. But in more advanced cancers, TGFβ has an oncogenic effect having a pro-tumorogenic, promoting EMT, angiogenesis and evasion of immune system. Also in distant metastasis, TGFβ enhances the extravasation and colonization of new organ by metastatic tumor cells. From (Blobe, Schiemann et al. 2000).

TGFβ AND TUMOR IMMUNE SURVEILLANCE

The role of TGFβ as an immune suppressor has been described for many years (Letterio and Roberts 1998; Yingling, Blanchard et al. 2004; Akhurst and Hata 2012). First evidences came from experiments of genetic disruption of TGFβ, which result in multifocal inflammation, pointing out the relevance of TGFβ as an immune suppressor (Shull, Ormsby et al. 1992; Kulkarni, Huh et al. 1993).TGFβ secretion by tumor cells or cells from the microenvironment can suppress the antitumor immune response leading to tumor escape and increase of the tumor promotion. TGFβ is a key enforcer of immune tolerance, and tumors that produce high levels of this cytokine may be shielded from immune surveillance. TGFβ inhibits Natural Killer cytototxicity and chemotaxis as well as it decreases CD8+ and CD4+ T cell proliferation and activation. It also decrases antigen presentation by macrophages and dendritic cells. TGFβ potentiates the activity of Treg and Th17 cells which are immune modulators (Figure 1.19).

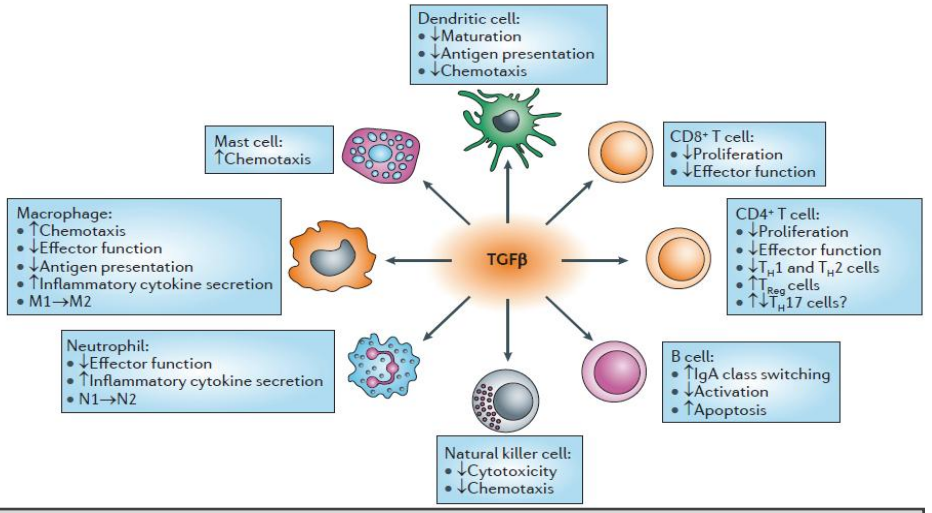


Figure 1.19. The immune suppressive role of TGFβ. TGFβ inhibits macrophages, Natural Killers and T lymphocytes and also activates Treg lymphocytes and Th17. Adapted from (Akhurst and Hata 2012).

TGFβ CONFERS CHEMORESISTANCE AND RADIORESISTANCE

TGFβ plays an important role in the response of tumor cells to conventional therapies such as chemotherapy or radiotherapy (Teicher 2001). It has been demonstrated by different authors that TGFβ overexpression confers drug resistance, both *in vitro* and *in vivo* (Teicher, Holden et al. 1996; Teicher, Ikebe et al. 1997). This suggests that blockage of TGFβ signaling pathway can sensitize tumor cells (Ohmori, Yang et al. 1998). First evidences of the role of TGFβ in response to ionizing radiation, comes from the observation that TGFβ is activated in irradiated tissues, presumably because the latent TGFβ complex has a specific redox sensitive conformation which is activated by reactive oxygen species generated by radiation (Jobling, Mott et al. 2006). It has been described that circulating TGFβ1 levels are increased after ionizing irradiation through activation of AP-1 transcription factor (Martin, Vozenin et al. 1997; Dancea, Shareef et al. 2009). This is correlated with more metastasis in a pre-clinical mouse model, and can be reverted by TGFβ blocking antibodies (Biswas, Guix et al. 2007; Massague 2008). Furthermore, radiation sensitivity of different tumor cell lines is increased when treated with a small-molecule inhibitor of the TGFβRI or a TGFβ neutralizing antibody (Kim, Lebman et al. 2003; Hardee, Marciscano et al. 2012). Interestingly, it has been recently reported that treatment with a small-molecule TGFβRI inhibitor LY2109761, can increase radiosensitivity in glioma. In this study, authors show that treatment with TGFβRI inhibitor potentiate radiation effect, reducing tumor growth, invasion, tumor microvessel formation and attenuating mesenchymal transformation in an *in vivo* pre-clinical model (Scheel, Eaton et al. 2011; Zhang, Kleber et al. 2011).

These results may suggest a new combinational therapy that may be more efficient in the treatment of glioma, with the concomitant inhibition of TGFβ pathway in combination with chemotherapy and/or radiotherapy (Zhang, Herion et al. 2011).

TGFβ IS AN ONCOGENIC FACTOR IN GLIOMA

Focusing in glioma, it has been demonstrated that TGFβ has an important oncogenic role. TGFβ pathway is very active in glioma and has been associated with poor clinical outcome in this deadly disease (Figure 1.19A) (Bruna, Darken et al. 2007).

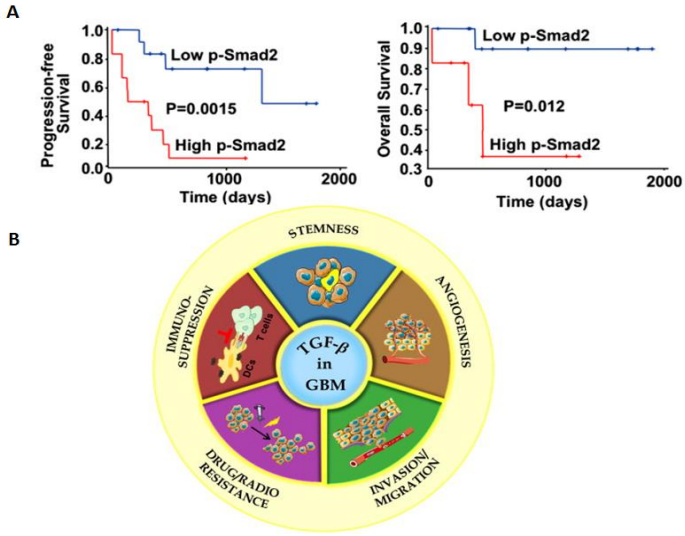


Figure 1.20. The TGFβ is an oncogenic factor in glioma. **A**. High TGFβ-pathway activity, measured by high levels of p-Smad2, correlates with poor progression-free survival and overall survival in GBM patients. Extracted from (Bruna, Darken et al. 2007). **B.** Diagram showing the oncogenic roles of TGFβ in GBM. TGFβ is involved in many critical aspects of GBM such as stemness, angiogenesis, invasion, migration, chemo and radioresistance and immunosupression. Adapted from (Joseph, Balasubramaniyan et al. 2013)

Many of the common features of GBM including cell proliferation, invasion of normal brain parenchyma, hypoxia, angiogenesis and suppression of immune system are related with the activation of the TGFβ pathway (Figure 1.20B) (Joseph, Balasubramaniyan et al. 2013). For example, it has been described that TGFβ promotes proliferation of glioma cells through the induction of PDGFB (Figure 1.21) (Bruna, Darken et al. 2007). TGFβ induces the expression of *PDGFB* in different patient-derived samples, only when the *PDGFB* promoter is not methylated. In those cases, there is a correlation between TGFβ activity (measured by the phosphorilation levels of Smad2), PDGFB expression and proliferation index measured by Ki67 staining (Figure 1.20 A and B). TGFβ increases glioma cell proliferation *in vitro*, and this is mediated by PDGFB secretion. Blockage of PDGFB either with a neutralizing antibody, or by a short hairpin RNA or with a specific inhibitor, causes a decrease in cell proliferation (Figure 1.21 C, D and E).

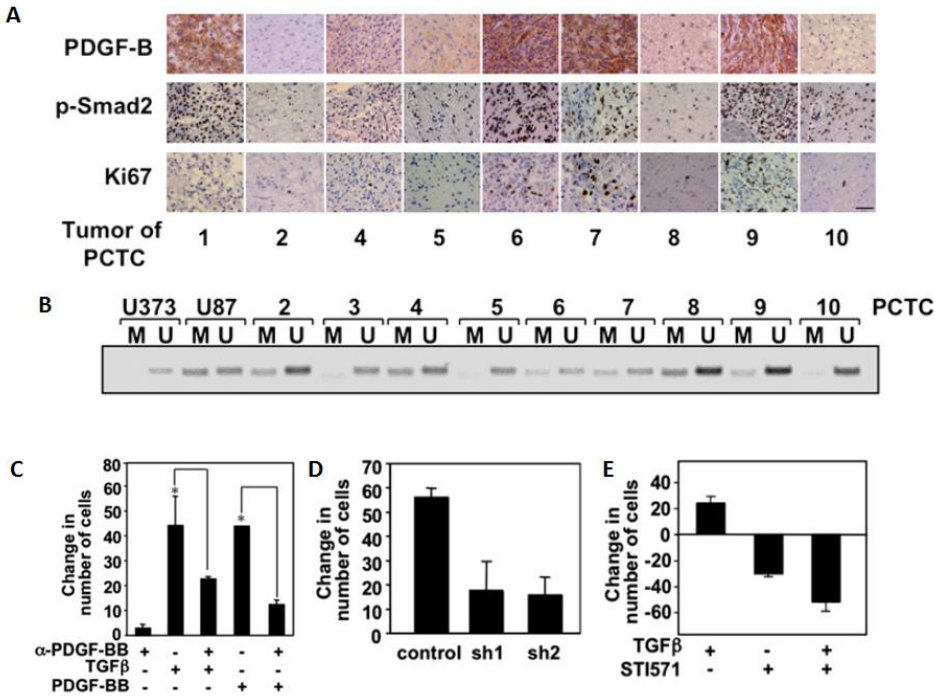


Figure 1.21. TGFβ induces expression of *PDGFB* and proliferation in GBM. **A**. In different patient-derived samples, high TGFβ activity (p-Smad2) correlates with PDGFB expression and proliferation (Ki67 staining), only in those tumors where PDGFB gene is unmethylated (**B**). **C**. Treatment of GBM cells with TGFβ or PDGFB increases proliferation, and it is decreased by anti-PDGFB blocking antibody. **D**. Knock-down of *PDGFB* by short hairpin RNA leads to a decrease in glioma cell proliferation, as well as treatment with a specific PDGFB inhibitor (**D**). Extracted from (Bruna, Darken et al. 2007).

TGFβ MAINTAINS CANCER INITIATING CELLS CHARACTERISTICS

TGFβ has also a critical role in maintaining the stem cell-like properties of certain cancer-initiating cells, including glioma initiating cells, breast-cancer initiating cells and leukemia-initiating cells in chronic myeloid leukemia (Mani, Guo et al. 2008; Penuelas, Anido et al. 2009; Seoane 2009; Naka, Hoshii et al. 2010). As discussed earlier, GICs are thought to be responsible for tumor initiation, progression and relapse of the disease. TGFβ increases the self-renewal of GICs by the induction of the cytokine LIF which is crucial to maintain GICs self-renewal and undifferentiated state (Penuelas, Anido et al. 2009). Treatment of patient-derived neurospheres with TGFβ or LIF increases GICs self-renewal, and this can be blocked by inhibiting LIF-JAK-STAT3 pathway pharmacologically (with P6 which inhibits JAK-STAT3 activity) or with anti-LIF blocking antibodies (Figure1.23 A and B and C). LIF is also crucial to maintain GICs stemness markers such as Musashi-1 (Msh-1), Sox2 or Nestin (Figure 1.23 D and E). It has also been demonstrated that TGFβ maintains GICs self-renewal and stemness through the Sox2-Sox4 axis (Figure 1.22) (Ikushima, Todo et al. 2009). In our project, we also demonstrate that TGFβ is critical to maintain GICs through the induction of *ID1* (Anido, Saez-Borderias et al. 2010)

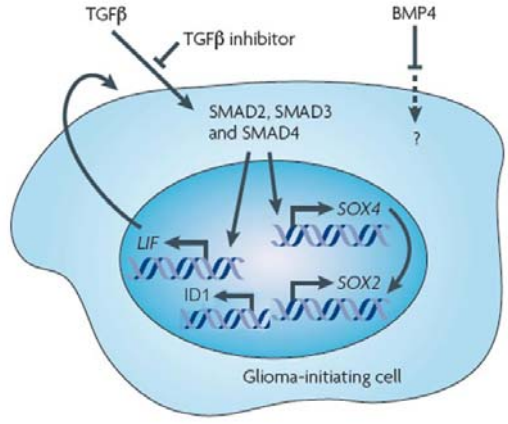


Figure 1.22. TGFβ maintains Glioma-initiating cells characteristics, through the induction of *SOX4-SOX2* axis and *LIF*. From (Ikushima and Miyazono 2010).

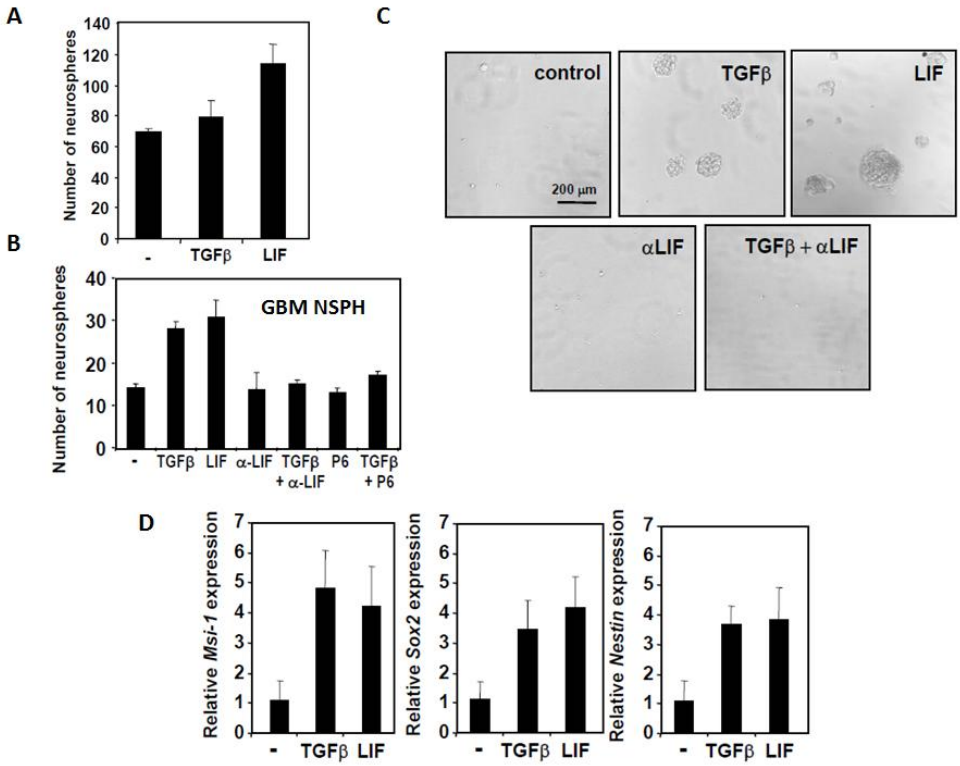


Figure 1.23. TGFβ increases self-renewal of GICs through the induction of *LIF*. **A**. Treatment of patient-derived neurospheres with TGFβ or LIF increases self-renewal of GICs. **B**. TGFβ increases self-renewal of GICs, but this can be blocked by inhibiting LIF signaling pathway with P6, an inhibitor of the JAK-STAT pathway. **C**. Representative images are shown. **D**. TGFβ and LIF increase the expression of different stemness-related genes such as *MUSASHI-1*, *SOX2* and *NESTIN*. From (Penuelas, Anido et al. 2009).

4. INHIBITION OF THE TGFβ PATHWAY AS A THERAPEUTIC STRATEGY IN GLIOMA

As described in previous chapter, there is growing clinical evidence that TGFβ have an important oncogenic role inducing tumor proliferation, invasion and metastasis, and promoting immune suppression. This succinct a special interest in blocking TGFβ as a new therapeutic approach (Korpal and Kang 2010). Some anti-TGFβ compounds have been developed and show efficacy in preclinical studies and clinical trials (Arteaga 2006; Bierie and Moses 2006; Wrzesinski, Wan et al. 2007; Seoane 2008; Ganapathy, Ge et al. 2010; Akhurst and Hata 2012).

Within the strategies developed to inhibit the TGFβ pathway there are inhibitors of TGFβ secretion (antisense oligonucleotides) that are delivered directly to the tumor. Trabedersen (AP1-2009) is an anti-TGFβ2 antisense RNA which is nowadays in clinical trials for GBM patients (NCT00761280) (Hau, Jachimczak et al. 2007; Akhurst and Hata 2012). There are also compounds blocking the ligand-receptor interaction, as for example, anti-TGFβ antibodies. Of note is the novel compound Fresolimumab (GC-1008) which is a TGFβ1, 2 and 3 blocking antibody in clinical trials phase I/II (NCT01112293). The TGFβ blocking antibody 1D11 has been tested in pre-clinical models and has shown promising effects in cancer treatment *in vivo* by preventing metastasis and decreasing radioresistance (Biswas, Guix et al. 2007). The blockade of the TGFβ interaction to its receptor decreases tumor cell viability and metastatic potential *in vivo* (Muraoka, Dumont et al. 2002). Recently, a TβRII-blocking antibody has been developed and (IMC-TR1)

and has just entered clinical trials for breast and colon cancer (NTC01646203) (Zhong, Carroll et al. 2010). Another strategy to pharmacologically block the TGFβ pathway activity is the small-molecule inhibitors that suppress the activity of the TGFβ Receptor Kinase (Yingling, Blanchard et al. 2004; Akhurst 2006). These compounds are ATP mimetics that competitively bind within the hydrophobic ATP binding pocket of the receptor kinase and prevent the phosphorilation of R-Smads and the activation of the pathway. Initial reports blocking TβRI activity used the small-molecule SB-431542, which is a potent inhibitor of the TGFβ pathway activity and demonstrate to be effective preventing tumor cell growth *in vitro* and *in vivo* (Hjelmeland, Hjelmeland et al. 2004; Halder, Beauchamp et al. 2005). Some of these inhibitors such as LY2157299, have entered clinical trials for efficacy in different types of cancers including glioma, pancreatic cancer, hepatocellular carcinoma and breast cancer (Glioma clinical trials: NCT01682187, NCT01582269, NCT01220271; Hepatocellular carcinoma clinical trial: NCT01246986; Pancreatic Cancer clinical trial: NCT01373164). Inhibition of TGFβ Type I receptor kinase by small molecules have shown anti-tumoral effect *in vitro* and *in vivo*, by decreasing cell motility, invasion and distant metastasis (Ehata, Hanyu et al. 2007; Ganapathy, Ge et al. 2010). Given the fact that TGFβ exerts a strong immunosuppressive effect in some tumors such as glioma, melanoma and renal cell carcinoma, treatment with TGFβ inhibitors may empower the immune system against the tumor (Yingling, Blanchard et al. 2004; Bierie and Moses 2006; Massague 2008; Akhurst and Hata 2012; Joseph, Balasubramaniyan et al. 2013).

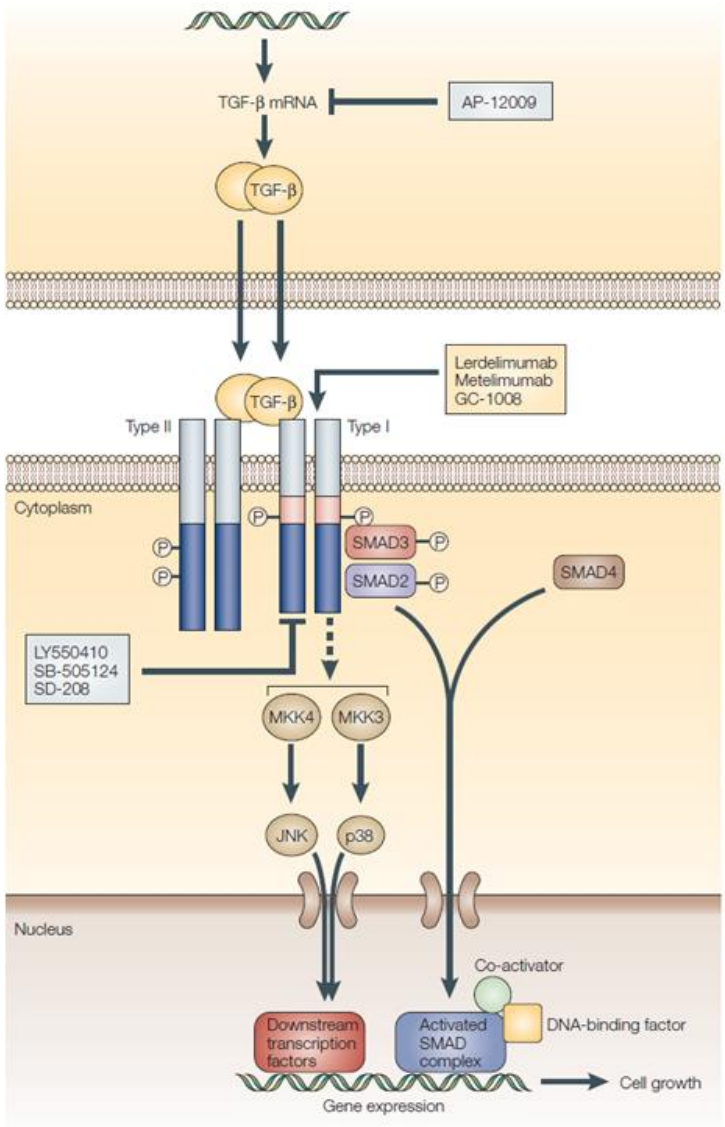


Figure 1.24. The TGFβ pathway as a therapeutic target. Given its important oncogenic role, TGFβ pathway is a target for pharmacological inhibition. TGFβ secretion can be blocked by antisense oligonucleotides. TGFβ binding to its receptor can be abolished by specific antibodies. TβRI Ser/Thre kinase activity can be repressed by small molecules that bind to ATP-binding site, inhibiting its enzymatic activity, thus blocking the activation of the pathway. Adapted from (Yingling, Blanchard et al. 2004)

5. *RUNX1* FAMILY OF TRANSCRIPTION FACTORS IN CANCER

RUNX TRANSCRIPTION FACTORS

The *RUNX* (Runt-related transcription factors) family of genes are also known as Acute Mieloid Leukaemia (AML), core-binding factor α (CBFα) or Polyoma Enhancer Binding Protein 2α (PEBP2α) (Jakubowiak, Pouponnot et al. 2000). These genes have such a diverse names due to its coincidental discovery as factors that bind to viral enhancers and as targets for chromosomal translocation in human leukemia, although the most used name Runx1 comes from its homolog in drosophila *Runt* which is essential for early embryonic segmentation (Gergen and Butler 1988; van Wijnen, Stein et al. 2004).

The three mammalian *RUNX* genes are part of an ancient 500kb triplication of chromosomes 1p, 6p and 21q (Strippoli, D'Addabbo et al. 2002). The 128 amino-acid N-terminal Runt domain is much conserved between the different isoforms. This domain is responsible for DNA and β chain cofactor (CBFβ) subunit interaction (Nagata, Gupta et al. 1999; Werner, Shigesada et al. 1999; van Wijnen, Stein et al. 2004). Each of the three Runx isoforms is transcriptionally regulated by two distantly located promoter regions, P1 (distal) and P2 (proximal) which results in differences in N-terminal sequences (Ghozi, Bernstein et al. 1996; Levanon and Groner 2004). The diversity of Runx variants is increased by further exon skipping and alternative 3' exon use (Miyoshi, Ohira et al. 1995; Levanon, Bernstein et al. 1996). Although the N-terminal Runt domain is the most conserved among different isoforms, there are also some conserved motifs in the C-terminal domain, including the VWRPY sequence which is required for

interaction with transcriptional co-repressors (Levanon, Negreanu et al. 1994) (Figure 1.25).

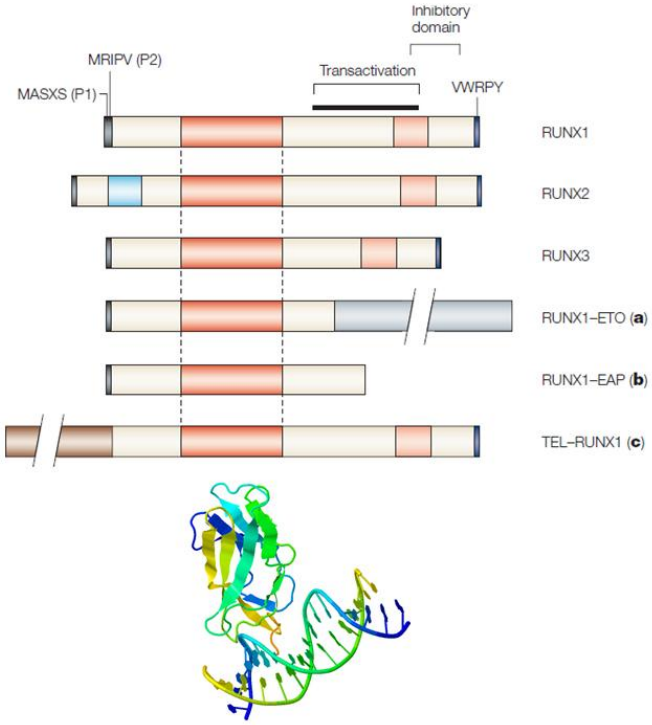


Figure 1.25. The Runx family of Transcription Factors. Runx1, Runx2 and Runx3 share the Runt N-terminal domain (in red), the transactivation domain and the VWRPY sequence in its carboxy-terminal domain. The most frequent translocations are represented: RUNX1-ETO (**a**) is the result of t(8:21) translocation and generates a fusion of the Runx N-terminal containing Runt domain with ETO negative regulator of transcription, causing a protein with a dominant negative effect. There is a truncated shorter version of Runx1 containing only the N-terminal domain which is also a dominant negative with DNA binding capacity but no transactivation capacity (**b**). TEL-RUNX1 is also the result of a common translocation t(12:21), generating a protein with TEL, an ETS transcription factor. Lower panel: 3D modeling of the RUNT domain binding to the DNA sequence. Adapted from (Blyth, Cameron et al. 2005)

Runx proteins bind directly to a conserved nucleotide sequence **R/TACCRCA**. The binding affinity is increased by the presence of **CBFβ** and is regulated by the presence of other binding cofactors. Runx proteins can bind and recruit a range of co-activators or co-repressors and regulate many different transcriptional responses in a context dependent manner (Figure 1.26).

The activity and stability of Runx proteins are influenced at post-translational level by phosphorylation, acetylation and sumoylation (Tanaka, Kurokawa et al. 1996; Imai, Kurokawa et al. 2004; Yamaguchi, Kurokawa et al. 2004).

Runx are very studied as key regulators of hematologic differentiation (de Bruijn and Speck 2004; Kurokawa 2006; Chen, Yokomizo et al. 2009; Swiers, de Bruijn et al. 2010).

RUNX1 KNOCK-OUT MICE

The importance of *RUNX1* in haematopoiesis has been demonstrated by the effect on knock-out mice. Mice embryos with homozygous deletion on *RUNX1* gene died at day E12.5, due to a lack of fetal liver hematopoiesis (Okuda, van Deursen et al. 1996). Mice also presented severe hemorrhaging in the central nervous system (CNS) (Wang, Stacy et al. 1996)

RUNX/AML IN CANCER

Runx transcription factors were initially identified as a part of a common translocation in Acute Myeloid Leukemia, involving the rearrangement of chromosome 8 and chromosome 21. This translocation t(8:21) generates a fusion protein containing the Runt

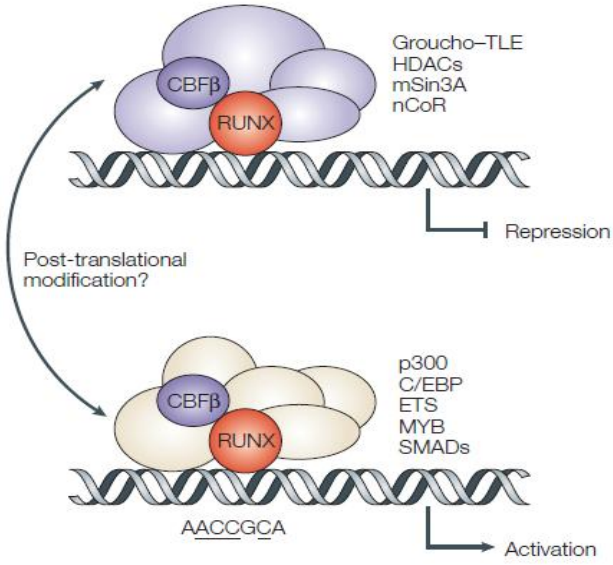


Figure 1.26. Runx transcription factors can act as repressors or activators of gene expression. Depending on the recruitment of cofactors and activators or repressors of transcription, Runx proteins have a different impact on gene expression. From (Blyth, Cameron et al. 2005)

DNA binding domain fused to ETO transcriptional repressor (also known as MTG8 or CBFA2T1) generating a Runx1 dominant negative form (Figure 1.25) (Miyoshi, Shimizu et al. 1991; Meyers, Lenny et al. 1995). Runx1 (AML1) is required for normal hematopoiesis and its disruption is one of the main causes of Acute Myeloid Leukemia.

There are also other translocations involving *RUNX1* gene, for example RUNX1-EAP which encodes the intact *RUNX1* N-terminus but with a premature truncation or short out-of-frame coding sequence. This results in a shorter Runx1 isoforms with ability to bind DNA sequence

but without the ability to recruit co-activators and co-repressors and thus lacking the transcriptional activity (Figure 1.25).

RUNX1 is also found mutated in about 5-10% of *de novo* leukemia and in up to 40% of therapy related leukemia (Osato, Asou et al. 1999; Harada, Harada et al. 2003; Christiansen, Andersen et al. 2004). *RUNX1* has been recently reported to be mutated or deleted in some breast cancers (Banerji, Cibulskis et al. 2012; Ellis, Ding et al. 2012), as well as in esophagus cancer (Dulak, Schumacher et al. 2012) , suggesting a possible role as a tumor suppressor for *RUNX1* in those patients (Taniuchi, Osato et al. 2012)

RUNX3 (AML2/CBFA3/PEBP2αC) is considered a tumor suppressor gene, as its loss of function has been related to gastric cancer. RUNX3 gene is subject of methylation, hemizygous deletion and point mutation in gastric carcinomas (Li, Ito et al. 2002).

However, there is growing evidence that effects of *RUNX* mutations and translocations are lineage restricted.

The clearest evidence that *RUNX* genes can act as **oncogenes** came from the finding that *RUNX* genes have been identified as common insertion sites for murine leukemia virus (MLV) in hematopoietic tumors. Insertions and hyperactivation of *RUNX* transcription factors by high-throughput screens are found as a cause of T or B-cell lymphomas (Mikkers, Allen et al. 2002; Suzuki, Shen et al. 2002; Wotton, Stewart et al. 2002) (Figure 1.27).

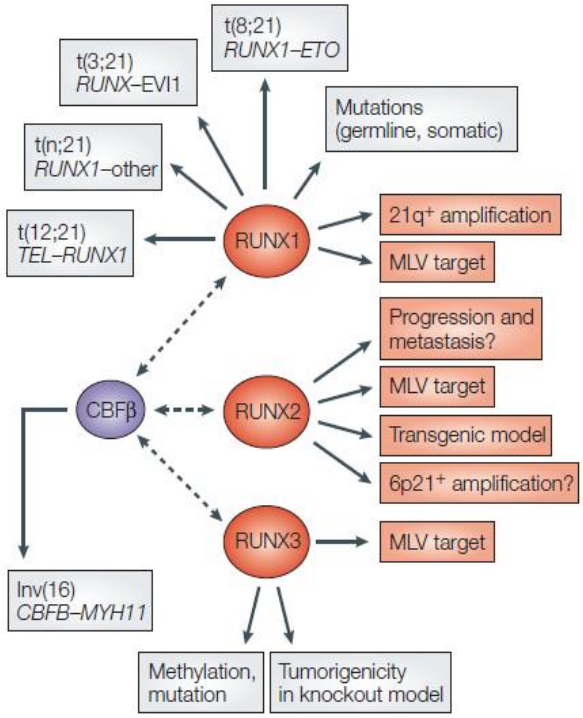


Figure 1.27. Runx transcription factors can act as oncogenes or tumor suppressors depending on the cellular context. In red, evidences supporting the role of Runx as dominant oncogenes. In grey, evidences supporting the role of Runx as tumor suppressors. From (Blyth, Cameron et al. 2005)

Evidences supporting the role of **Runx1** as an oncogene come from B-ALL and myeloid leukemia in which a large segment of chromosome 21q (10Mb) is amplified. *RUNX1* gene is within the chromosome 21 region amplified in Down's syndrome, and they are prone to leukemia (Hasle, Clemmensen et al. 2000; Niini, Kanerva et al. 2000; Robinson, Broadfield et al. 2003). High expression of Runx1 has also been found in the absence of gene amplification, indicating other mechanisms of deregulation (Mikhail, Serry et al. 2002).

Ectopic overexpression of *RUNX1* in mouse embryonic fibroblasts (MEFs) cause a transformed phenotype but only in the absence of functional p53. In contrast, wild-type MEFs expressing Runx1 in a functional p53 background undergo senescence-like growth arrest (Wotton, Blyth et al. 2004).

Runx1 is widely studied as a human haematopoietic stem cell factor, but its role in solid tumors has now beginning to be understood. Runx1 has been studied in many solid tumors, especially in epithelial tumors. Metanalysis of Oncomine gene-expression data shows that Runx1 is overexpressed in a significant fraction (47 out of 138 studies) and only underexpressed in 5 studies of human solid tumors, especially in epithelial cancers (Figure 1.28) (Scheitz, Lee et al. 2012). Different carcinogenesis experiments have shown that Runx1 is required for tumor initiation but not for tumor promotion. It is also shown that Runx1 is important for tumor mainteinance, as Runx1 depletion leads to tumor regression (Scheitz, Lee et al. 2012).

Further evidences of Runx1 oncogenic role have been recently reported in solid-tumors, as it has been identified as one of the most highly over expressed genes in a microarray of invasive endometrial

carcinoma (Planaguma, Diaz-Fuertes et al. 2004; Planaguma, Gonzalez et al. 2006).

Runx1 transcription factor has been recently included in the six-gene signature of transcription factors that drive mesenchymal subtype of GBM (see Figure 1.7) (Carro, Lim et al. 2010). This suggests that Runx1 may be having an important oncogenic role also in GBM.

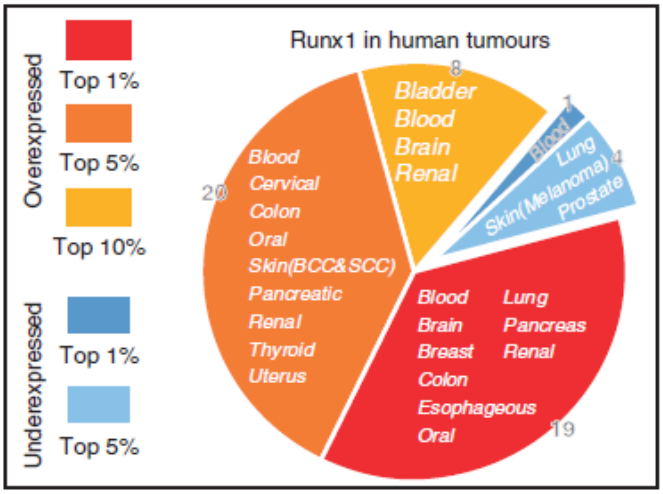


Figure 1.28. *RUNX1* expression in different human cancers. *RUNX1* is found overexpressed in 30% of human tumors including different skin cancers, breast, oesophageal, lung, brain, colon and pancreatic cancers (red-orange coloured parts of the chart), and is only down-regulated in 3% of cancers. Of note, in almost 14% of cancers, Runx1 is in the top 1% overexpressed genes. Data extracted from Oncomine (Scheitz, Lee et al. 2012).

There are also evidences of oncogenic role for **Runx2** isoform. Runx2 over expression in combination with Myc and loss of p53, drive proliferations *in vivo* (Blyth, Terry et al. 2001). Further evidence of Runx2 as an oncogene comes from ectopic expression in osteoblasts

and endothelial cells, where the effect is similar to oncogenic transformation, including enhanced cell migration, invasion, survival and angiogenesis (Sun, Vitolo et al. 2001). Of note is the regulation of Runx2 by phosphorylation through different signaling pathways such as PI3K, PKC and MAPK (Xiao, Jiang et al. 2002; Franceschi, Xiao et al. 2003; Kim, Kim et al. 2003; Fujita, Azuma et al. 2004; Qiao, Shapiro et al. 2004). Runx2 has been implicated in metastasis to the bone (Selvamurugan, Kwok et al. 2004).

On the other hand, **Runx3** is mainly described to act as a tumor suppressor in some epithelial cancers, especially in gastric cancer (Guo, Weng et al. 2002; Chi, Yang et al. 2005; Ito, Liu et al. 2005; Yanada, Yaoi et al. 2005; Yano, Ito et al. 2006). There are some reports indicating a potential oncogenic role for Runx3, for example in pancreatic cancer (Li, Kleeff et al. 2004).

The expression of 3 Runx isoforms is not uniform in all tissues. This opens the question if they can have some compensatory effects, and if their role as oncogenes or tumor suppressors might be different depending on the tumor type. This complexity in Runx transcription factors highlights the importance of cross-talks and interactions with other pathways. Runx proteins are at the core of many different signaling pathways and they are important for the cross-talk between them. In Figure 1.29 is shown that Runx transcription factors can be regulated by several pathways and they are involved in many cellular processes such as cell cycle regulation, hematopoietic differentiation, bone development or metastasis. Such pleiotropic effect is what gave them this duality, acting as both, tumor suppressors or oncogenes depending on the cellular context.

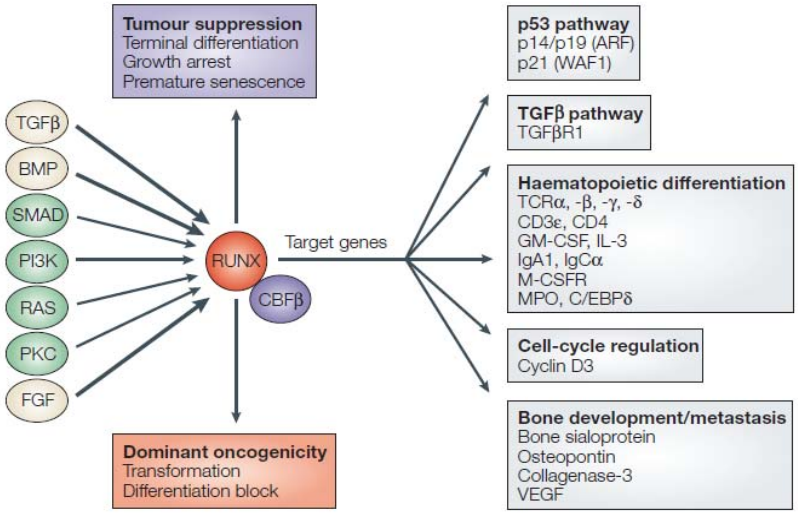


Figure 1.29. Runx transcription factors are at the core of many different signaling pathways. They can be activated by different pathways such as BMP, TGFβ, RAS, PI3K, PKC or FGF. In turn, they are involved in many important cellular processes. They are important for the cross-talk between different pathways. They have a dual role acting as tumor suppressors or dominant oncogenes depending on the cellular context. From (Blyth, Cameron et al. 2005)

RUNX AND TGFβ PATHWAY

We have previously discussed the duality of TGFβ in cancer, which can both inhibit the growth of normal cells and induce a more aggressive phenotype in cancer cells. Here we discuss some evidences for the involvement of Runx factors in the TGFβ pathway.

Runx1 (AML1) is described to physically bind to Smads. Each of the R-Smads interacts directly with each of the tree members of the Runx Transcription factors (Figure 1.25). This two transcription factors are known to cooperate in some transcriptional responses, such as the Immunoglobulin A (IgA) class switching by TGFβ (Hanai, Chen et al. 1999; Pardali, Xie et al. 2000; Zhang and Derynck 2000). This interaction involves the MH2 domain of Smads and multiple regions in Runx1 protein (Pardali, Xie et al. 2000). Recently, some authors have pointed out that Runx1 is a co-activator together with FoxO3 of the TGFβ-mediated induction of *BIM* (Wildey and Howe 2009). It has been also reported that Runx expression is required to recruit Smads to subnuclear sites of active transcription (Zaidi, Sullivan et al. 2002).

The Runx1-Smad complexes seem to be formed constitutively in the cytoplasm, becoming active by association with additional factors in the nucleus in response to TGFβ pathway activation (Pardali, Xie et al. 2000). Consistently with this cooperation between TGFβ and Runx/AML, AML1-ETO dominant negative fusion protein negatively regulate TGFβ pathway activity, suggesting that AML1/Runx1 may be a key mediator of the TGFβ signaling pathway (Jakubowiak, Pouponnot et al. 2000).

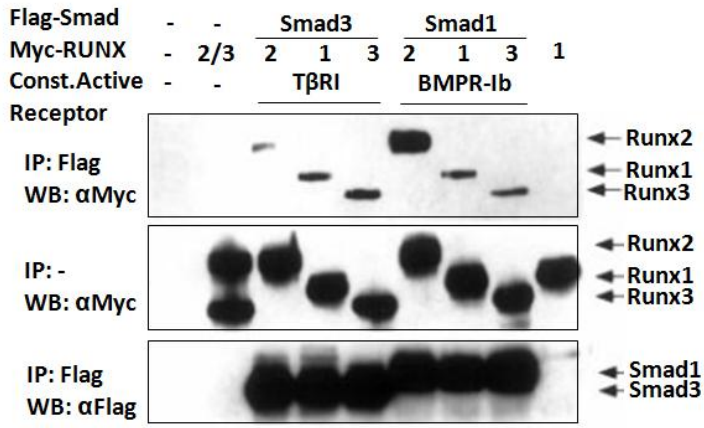


Figure 1.30. Runx transcription factors bind to different Smads upon TGFβ or BMP pathway activation. Flag-Smad 3 (TGFβ pathway) or Smad1 (BMP pathway) were co-transfected with different RUNX isoforms with MYC tag in the presence of TGFβRI or BMPR-Ib to activate the pathway. Smads were immunoprecipitated with a Flag resin and immunoblott was performed with anti-Myc antibodies to detect Runx different isoforms. Adapted from (Hanai, Chen et al. 1999).

The interaction between Runx and TGFβ is more complex, as TGFβ has been described to activate *RUNX* genes at the transcriptional and post-transcriptional level, promoting the activation or stabilization of Runx protein (Ito and Miyazono 2003). Furthermore, Runx1 is involved in the regulation of TβRI, thus enhancing the capacity of the cells to respond to TGFβ (Ito and Miyazono 2003; Miyazono, Maeda et al. 2004).

At this time point, there are not many targets known to be regulated by the cooperation between Runx1 and Smad transcription factors. In this project we will try to demonstrate that Runx1 is indeed a key mediator of the TGFβ oncogenic effect in glioma, cooperating with Smads in the induction of many transcriptional responses.

OBJECTIVES

The aim of this thesis is the **study of the molecular mechanisms implicated in the oncogenic effect of the TGFβ pathway**, specially focused in glioma.

The main objectives are listed below:

- Study and characterize Glioma Initiating Cell (GIC) population and define biomarkers to isolate them
- Study the effect of the TGFβ on GICs and the consequences of the TGFβ inhibition by selective compounds that are being developed in the clinic
- Characterize the source of TGFβ in glioma and study the role of the tumor microenvironment
- Study the mechanisms of resistance of GICs to conventional therapies (i. e. radiotherapy) and the role of TGFβ as a mechanism of radioresistance
- Overcome radioresistance of GICs by the combination of conventional therapies (such as radiotherapy) and targeted therapies
- Further characterize the mechanism of LIF induction by TGFβ, especially focusing in the role it may have in GICs
- Find new mediators of the TGFβ oncogenic effect and possible biomarkers of response to TGFβ inhibition treatments
- Analyze patient-derived samples (*in vitro* and *in vivo*) to validate our findings

MATERIALS AND METHODS

1. *IN VITRO* TECHNIQUES

1.1. MOLECULAR CLONING

Constructs

ID1 overexpression

ID1 cDNA was kindly provided by Dr. Francesc Ventura from IDIBELL, Barcelona, Spain.

ID1 and ID3 knock-down

Lentiviral pGIPZ vectors with a microRNA targeting *ID1* and *ID3* were purchased from Open Biosystems (Thermo Scientific, Walham MA, USA).

LIF promoter

A firefly-luciferase reporter vector was used to study the induction of LIF at the molecular level. *LIF* promoter region -276/+32 was cloned between SacI and NheI into pGL2-basic luciferase vector (Promega) as described in (Penuelas, Anido et al. 2009).

Smad binding element (SBE) and Runx1 binding site were mutated by PCR-directed mutagenesis.

The primers used were the following:

LIF promoter (-276/+32):

F: 5'-GCCCGAGCTCCGGGACAAGCCAGGCAGGAAAAC-3'

R: 5'-GCCCGAGCTCCGGGACAAGCCAGGCAGGAAAAC-3'

LIF mutant Runx1 Binding Site:

F: 5'- CCATTCATAATTTCCTATGATGCCCCGGGAACAACTTCCTGGACTG-3'

R: 5'-CAGTCCAGGAAGTTGTTCCCGGGGCATCATAGGAAATTATGAATGG-3'

Briefly, PCR was performed using PFU-Turbo polymerase (Stratagene, La Jolla, CA, USA) and then, template DNA was digested using DpnI restriction enzyme (Roche Diagnostics, Basel Switzerland). After this, mutated new generated DNA was transformed into competent DH5α E-Coli (Promega, Madison, Wisconsin, USA) and grown in Ampicillin- Lysogeny Broth (LB) agarose plates. Single colonies were grown in LB with Ampicilin and DNA was extracted using a mini-prep kit (GeneService, Cambridge, UK). Purified constructs were checked by digestion with specific restriction enzymes and mutation was confirmed by Sanger sequencing.

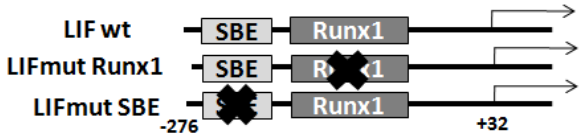


Figure 2.1. Schematic representation of LIF promoter wild type and mutant forms for Smad Binding Element and Runx1 binding site.

LIF promoter (TGFβ responsiveness region): -276/+32.

LIF SBE mutated: point mutations in -183 and -184.

LIF Runx1 mutated: 3 point mutations in -109, -106 and -105 base pairs.

```
LIFmut CACTCTCACTTTCTTCCATTCATAATTTCCTATGATGCCCCGGGAACAACTTCCTGGACT
       :::::::::::::::::::::::::::::::::::::::: ::   ::::::::::::::
LIFwt  CACTCTCACTTTCTTCCATTCATAATTTCCTATGATGCACCTCAAACAACTTCCTGGACT
```

Figure 2.2. Sequence of LIF promoter sequence wild type form and mutant for Runx1 binding site.

RUNX1 knock-down

Initially, short hairpin primers were designed and ligated into pRetroSuper vector (kindly provided from Dr Eichhorn from NKI, Netherlands)

The primers were the following:

Runx1 sh#1 F: 5'-
GATCCCCTCGAAGTGGAAGAGGGAAATTCAAGAGATTTCCCTCTT
CCACTTCGATTTTTGGAAA-3'

Runx1 sh#1 R: 5'-
AGCTTTTCCAAAAATCGAAGTGGAAGAGGGAAATCTCTTGAATTT
CCCTCTTCCACTTCGAGGG-3'

Runx1 sh#2 F: 5'-
GATCCCCGGCAAACTAGATGATCATTCAAGAGATGATCATCTAGT
TTCTGCCTTTTTGGAAA-3'

Runx1 sh#2 R: 5'-
AGCTTTTCCAAAAAGGCAGAAACTAGATGATTCATCTCTTGAATG
ATCATCTAGTTTCTGCCGGG-3'

Runx1 sh#3 F: 5'-
GATCCCCTCGAAGACATCGGCAGAAATTCAAGAGATTTCTGCCGA
TGTCTTCCATTTTTGGAAA-3'

Runx1 sh#3 R: 5'-
AGCTTTTCCAAAAATCGAAGACATCGGCAGAAATCTCTTGAATTT
CTGCCGATGTCTTCGAGGG-3'

Runx1 sh#4 F: 5'-
GATCCCCGGTCGAAGTGGAAGAGGGATTCAAGAGATCCCTCTTCC
ACTTCGACCTTTTTGGAAA-3'

Runx1 sh#4 R: 5'-
AGCTTTTCCAAAAAGGTCGAAGTGGAAGAGGGATCTCTTGAATCC

CTCTTCCACTTCGACCGGG-3’

Runx1 sh#5 F: 5’-

GATCCCCGGGAAAAGCTTCACTCTGATTCAAGAGATCAGAGTGAA

GCTTTTCCCTTTTTGGAAA-3’

Runx1 sh#5 R: 5’-

AGCTTTTCCAAAAAGGAAAAGCTTCACTCTGATCTCTTGAATCAG

AGTGAAGCTTTTCCCGGG-3’

For lentiviral infection, lentiviral pGIPZ vector with a microRNA targeting *RUNX1* was purchased from Open Biosystems (Thermo Scientific, Walham MA, USA).

An inducible lentiviral pTRIPZ vector with a Tet-ON system targeting *RUNX1* was purchased from Open Biosystems (Thermo Scientific, Walham MA, USA).

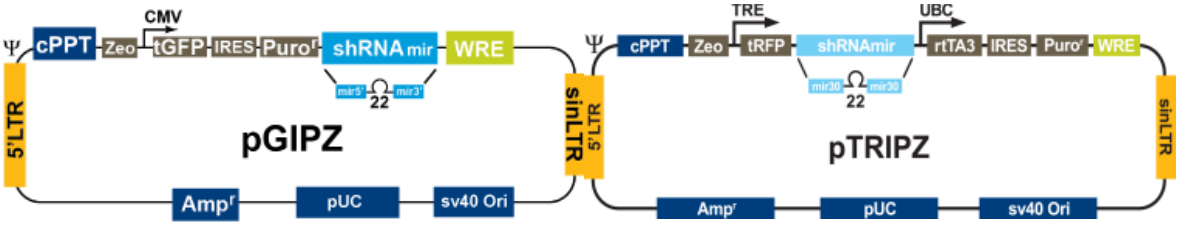


Figure 2.3. Maps of pGIPZ and pTRIPZ vectors purchased from Open Biosystems. pGIPZ contains a short-haripin RNA and pTRIPZ also contains a TET-On responsive element.

Runx1 overexpression

Runx1 coding sequence (1360bp) was PCR-amplified using PFU polymerase (Stratagene, La Jolla, CA, USA) with the following primers containing BglII and XhoI restriction sites:

Runx1Long F: 5'-CCCAGATCTATGCGTATCCCCG-3'

Runx1Long R: 5'-5'CCCCTCGAGTCAGTAGGGCC-3'

The amplified fragment was run in an agarose gel stained with Ethidium Bromide (Sigma Aldrich) and then purified using QIAEXII kit (Quiagen, Hilden, Germany) and ligated into an expression vector pCMV-flag (from Dr Seoane).

This was subcloned a posteriori into two retroviral vectors (pLPCX and pLNX2 with Puromycin and Neomycin resistance respectively). Runx1 was digested by SacI and XbaI restriction enzymes and ends were repaired using End-IT Repair Kit (Epicentre, Madison, WI, USA) and gel purified using QIAEXII kit (Quiagen) Hilden, Germany). The vectors were digested with StuI restriction enzymes which cut in blunt ends, de-phosphorilated using Alkaline Phosphatase from calf intestine (Roche Diagnostics, Basel, Germany) for 45min and gel-purified using QIAEXII kit.

For lentiviral expression, Runx1 coding sequence was PCR amplified using the following primers described in (Challen and Goodell 2010).

Runx1B F: 5'-CACCGATGCGTATCCCCGTAGATGCCAGC-3'

Runx1B R: 5'-GTCAGTAGGGCCTCCACACGGCCT-3'

This primers contain a CACC sequence at the 5' end of the primer that allowe the recombination into TOPO/pENTR vector (Invitrogen, Carlsbad

CA, USA). Using cell free Gateway ® LR Recombinase II cloning system (Invitrogen, Carlsbad CA, USA), Runx1 coding sequence was cloned into pLenti-CMV-Neo-DEST purchased from Addgene.

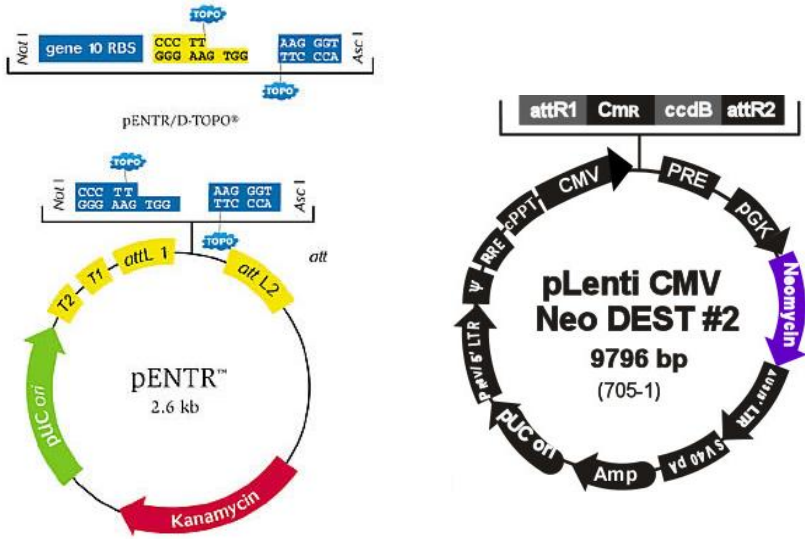


Figure 2.4. Schematic representation of vector TOPO/pENTR and pLenti-CMV-Neo DEST. Gateway clonning system allows to recombine a PCR product from TOPO/pENTR into pLentiDEST.

AML1-ETO (dominant negative form):

pCMV-AML1-ETO was purchased from Addgene (Cambridge, MA, USA). Then it was subsequently subcloned into pLNX2 with Neomicyn resistance, using HindIII and NotI restriction sites.

Luciferase lentiviral vector

pLenti-CMV-LUC constituvely expressing firefly luciferase was purchased from Addgene (Cambridge, MA, USA).

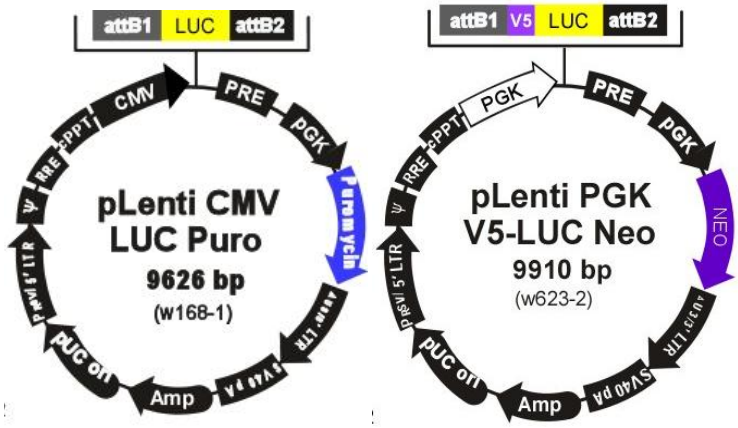


Figure 2.5. Schematic representation of vector pLENTI-CMV expressing luciferase, with Puromicyn resistance (left) or Neomycin resistance (right). These vectors are used to monitor tumor growth by *in vivo* imaging.

1.2. CELL LINES AND TISSUE CULTURE

Table 1: List of cell lines used

Cell line	Origin	CμLture media
293T-HEK	Human Embryonic Kidney	DMEM + 10% FBS
Phoenixϕ	Human Embryonic Kidney	DMEM + 10% FBS
293T-GP2	Human Embryonic Kidney	DMEM + 10% FBS
U373-MG	Glioblastoma	DMEM + 10% FBS
U87-MG	Glioblastoma	DMEM + 10% FBS
A172	Glioblastoma	DMEM + 10% FBS
T98G	Glioblastoma	DMEM + 10% FBS
C3	Glioblastoma	DMEM + 10% FBS
C4	Glioblastoma	DMEM + 10% FBS
C5	Glioblastoma	DMEM + 10% FBS
	Glioblastoma	DMEM + 10% FBS
4T1	Mouse breast	RPMI + 10% FBS
H1993	Lung adenocarcinoma	RPMI + 10% FBS
HUVEC	Human umbilical Vein	EGM media + 5% FBS + bFGF + EGF + Heparin + Ascorbic Acid
hCMEC	Human cerebral endothelium	EGM media + 5% FBS + bFGF + EGF + Heparin + Ascorbic Acid

Briefly, most of the cell lines were cultured in NUNC surface plates (Thermo Fisher Scientific, Waltham MA, USA) or BD plates (San Jose, CA, USA) using Dulbecco's Modified Eagle Medium (DMEM, purchased from GIBCO, Invitrogen) or Roswell Park Memorial Institute medium (RPMI), supplemented with 10% Fetal Bovine Serum (FBS, from GIBCO, Invitrogen), 20.000 units of Penicillin/Streptomycin (GIBCO, Invitrogen) and 250μg of Fungizone-AmphotericinB (GIBCO, Invitrogen) and Plasmocin (Invivogen,

San Diego CA, USA) Cells were maintained in a subconfluent state and were frozen with FBS and 10% DMSO.

Cells were maintained at 37º in an atmosphere of 5% CO2.

For endothelial cells, plates were pre-coated with Rat Collagen for 1hour at 37º before seeding the cells. Cells were maintained in a special medium EBM2 Basal Medium (Lonza, Basel Switzerland) supplemented with hEGF, Hydrocortisone, Gentamicin-AmphotericinB, FBS, VEGF, hFGF-B, IGF1, Ascorbic Acid and Heparin following manufacturer's instructions.

Mycoplasm detection tests were perfomed regulary to ensure there was no mycoplasm contamination of the cells.

Before reaching the confluence, cells were rinsed with PBS and incubated with Trypsin-EDTA (GIBCO, Invitrogen) for 5 minutes at 37º. Complete medium was added to inactivate trypsin and cells were subsequently diluted in fresh media.

Cells stably infected with retroviral o lentiviral plasmids were selected according to its resistance with Puromycin (1µg/mL, from Sigma Aldrich), Neomycin (G418, 700µg/mL, from Invitrogen).

1.3. ISOLATION AND CULTURE OF NEUROSPHERES FROM PATIENTS TUMORS

Tumor sample was collected right after surgery and rapidly (less than 30minutes) processed. Tumor pieces was chopped with a scalpel and digested using 500µL of DNAseI (500u/mL) (Sigma-Aldrich, San Louis – MO, USA) and 100µL of Collagenase (200u/mL) (Sigma-Aldrich) for 1hours (depending on the tumor piece) at 37º with 1000 rpm agitation.

After that, cells were filtered through a 70µm nylon cell strainer (BD Biosciences) and washed with abundant PBS. Cells were pelleted by centrifugation at 400g during 5 minutes and eritrocytes were lysed with Eritrocyte-Lysis Buffer for 4 minutes at room temperature. After that, cells were washed again with PBS and centrifuged at 400g for 5 minutes. Pelleted cells were resuspended in DMEM medium supplemented with 10% FBS for primary culture (PCTC) or with Neurobasal medium (both from GIBCO, Invitrogen) supplemented with B27 (GIBCO, Invitrogen), EGF and FGF (PeproTech, Rocky Hill NJ, USA), and the corresponding antibiotics and antimycotics.

Cells grown in Neurobasal medium form neurospheres which are enriched in glioma-initiating cells.

Neurospheres were maintained in Neurobasal medium and were disaggregated manually using a micropipette to avoid the formation of bigger aggregates of spheres. They were frozen using Bambanker cell freezing media (Lymphotech Inc, Tokyo Japan).

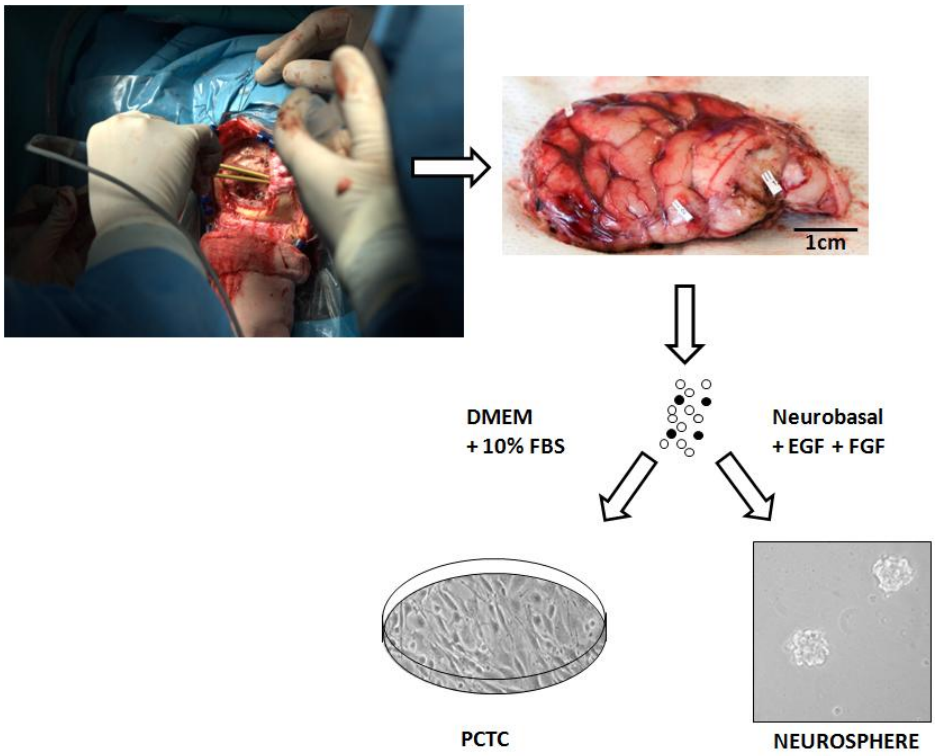


Figure 2.6. Generation of neurosphere cultures derived from patient's tumors. **A.** GBM resection from a patient. **B**. Cells are grown in DMEM with 10% FBS for a PCTC or **C**. Cells are grown with Neurobasal medium for neurospheres enriched in GICs.

1.4. *IN VITRO* TREATMENTS

Cells were treated with different cytokines or inhibitors as summarized here:

TGFβ1 (Peprotech) used at **100pM.**

hLIF (Millipore) used at **20ng/mL.**

TGFβ Receptor I inhibitor (LY210976) (from Eli-Lilly, Indianapolis, IN, USA) used at **2µM.**

TGFβ Receptor I inhibitor (LY215799) (Eli-Lilly) used at **2µM.**

anti-TGFβ blocking antibody (R&D Systems, Minneapolis, MN, USA) used at **1,25µg/mL.**

anti-LIF blocking antibody (made in our laboratory) used at **10µg/mL.**

Doxycycline: (Sigma Aldrich) used at **1µg/mL.**

Dymethil Sulfoxide (DMSO): (Sigma Aldrich) used as a vehicle of TGFβ inhibitor.

In vitro irradiation of cells

Cells were collected in 15mL polystyrene tubes (BD Bioscience, San Jose, CA, USA) full of media. Cells were placed in an ADAMS plastic support and were irradiated at a single dose of 9Gy in a Cobalt radioactive source.

1.5. CELL TRANSFECTION

Cells were transfected using 3 different protocols, depending on the requirements of the experiment.

a. Lipofectamine transfection

Cells were seeded at a sub-confluent state in 60mm or 100mm plates.

8µg of DNA were mixed with 24µL of Lipofectamine 2000 reagent (Invitrogen) with Optimem Medium (GIBCO, Invitrogen) in polypropylene tubes (BD Biosciences). Liposomal mixture was left for 20 minutes and afterwards added to the normal culture medium. After 16 hours cells were rinsed with PBS and fresh medium was added. For protein expression we waited for 24h after cells were lysed.

b. Calcium phosphate transfection

Cells were seeded the previous day at 70% of confluence in 150 cm plates. 1 hour prior to transfection, cells were treated with Chloroquine 25µM (Sigma Aldrich). 25µg of plamid DNA were transfected and then TE 0.1X (Tris1mM – EDTA 0.1mM pH 8.8) was added up to 1125µL. We added 125µL of CaCl2 2,5M and mixed well by pipetting. Then we added 2x HBS (NaCl 280mM – HEPES 100mM – Na2HPO4 1.5mM; 7.11<pH<7.13) drop wise continuously vortexing the tube. The mixture was immediately added to the cells for an over-night transfection. Next morning, cells were rinsed with PBS and fresh medium was added to the cells.

c. siRNA transfection

ON-TARGET plus SMARTpool siRNA was purchased from Dharmacon (Thermo Fisher, Laffayete, CO, USA) and

resuspended in the appropriate buffer according to manufacturer's instructions to obtain a 20µM dilution. 15µL of siRNA and the appropriate controls (Scrambled siRNA or siGlo) were mixed in 300µL of DMEM without growth factors. 12µL of Lipofectamine 2000 (Invitrogen) were added to 300µL of DMEM and mixed together by energically pipetting up and down. After 20 minutes, 900µL of 10%FBS DMEM were added and the mixture was added to the cells for an over-night transfection. Next morning cells were rinsed with abundant PBS and fresh medium was added. After 72 hours, cells were lysed for RNA o protein extraction.

For the Runx1 knock-down with siRNA, the sequences of siRNA used were the following:

J-003926-05: 5'-UGACAACCCUCUCUCGCAGA-3'

J-003926-06: 5'-GAACUAGAUGAUCAGACC-3'

J-003926-07: 5'-CGAUAGGUCUCACGCAACA-3'

J-003926-08: 5'-CAAAUGAUCUGGUGGUUAU-3'

1.6. VIRAL INFECTIONS

Retroviral infections

Retroviral plasmids were transfected into Phoenixϕ or 293T-GP2 cells which stably express GAG, POL and ENV viral genes (in the case of Phoenixϕ) or only GAG and POL in the case of 293T-GP2, which also need the co-transfection of the ENV gene (VSV-g).

We used the protocol of Lipofectamine transfection. After an over-night (16 hours) transfection, cells were rinsed and incubated with the appropriate medium. Medium containing viral particles was collected after 24 and 48 hours and filtered through a 45µm filter

using a syringe. Viral particles were incubated with the recipient cells for an over-night (16 hours) with 0.8µg/mL of Polybrene (Sigma Aldrich) added. Cells were centrifuged at 1800 r.p.m for 45minutes to enhance the infection.

Lentiviral infections

Lentiviruses are more effective infecting cells that are not dividing, for example stem cells. So we used this protocol when working with neurosphere cultures that do not divide as fast as immortalized cell lines.

We transfected low passage 293T cells with ENV (VSV-G or pMD2G) and PACKAGING (PAX2) vectors for lentiviral production, together with our lentiviral vector of interest. We used calcium transfection protocol. After an over-night (16 hours) transfection, 293T cells were carefully rinsed and incubated with the appropriate medium (for example Neurobasal medium if the cells to infect were neurospheres). Medium containing viral particles was collected after 24 and 48 hours and filtered through a 45µm filter (Millipore, Billerica, MA, USA) using a syringe. Cells were incubated with viral for an over-night (16 hours) with 0,8µg/mL of Polybrene (Sigma Aldrich) added.

1.7. RNA PURIFICATION AND QUANTITATIVE REAL-TIME PCR

RNA purification

For RNA extraction cells were treated (if necessary) for 3hours. Cells were rinsed with PBS and lysed in RLT buffer (from RNA extraction minikit – Qiagen). RNA was immediately extracted according to the manufacturer instructions, or RLT-lysed cells were frozen at -80º. RNA concentration and quality was assessed with a Nanodrop (Thermo Scientific).

For Laser-Captured Microdissected Samples or sorted samples with a very low RNA amount, we used the RNA extraction microkit (Qiagen) or Arcturus (Life Technologies). RNA yeld and quality was assessed with Pico or Nano-Chip from Agilent Technolgies (Santa Clara, CA, USA)

Quantiative Real-Time PCR

cDNA was generated using iScript cDNA synthesis kit from BioRad (Hercules, CA, USA) according to manufacturer's instructions.

To analyze gene expression, quantitative Real-Time PCR was performed using Applied Biosystem Taqman probes (Applied Biosystems – Life Technologies) and Taqman Real-Time PCR master mix (from Applied Biosystems – Life Technologies).

Real-time amplification was performed in 384 well clear plates in a final volume of 10µL using CFX384 Real Time System C1000 Touch

Thermal Cycler from BioRad. The program used for amplification was the following:

1. 50º for 2'
2. 95º for 10'
3. 95º for 15''
4. 60º for 1'
5. Go to 3 x 39 cycles

Results were analyzed using the ddCT method and normalized by the expression of an endogenous housekeeping gene (*GAPDH*, 18S or POLR2A) and by the control sample.

1.8. DNA PURIFICATION AND SEQUENCING

Total genomic DNA from cells was isolated using DNA micro and mini kit (Qiagen) according to manufacturer instructions. DNA concentration and quality was assessed by Nanodrop.

Plasmid DNA was isolated and purified from E-coli cultures using the QuickClean II Plasmid Miniprep kit (GenScript, Piscataway, NJ, USA). DNA was sequenced using the Sanger method with BigDye v1.1 and a specific primer for the region of interest.

For patient samples, DNA was extracted according to the protocol of DNA micro or mini kit (QIAGEN) depending on the total amount of tissue. A little piece of frozen tumor was used to obtain 100 ng of DNA. DNA was sequenced by high-throughput sequencing.

1.9. PROTEIN EXTRACTION, IMMUNOBLOTTING AND IMMUNOPRECIPITATION

Immunoblotting

Cells were lysed using RIPA buffer supplemented with protease inhibitor cocktail tablets (Roche Pharma, Schweiz, Switzerland) at 4º and centrifuged for 20 minutes at 4º and cell membranes were discarded. Protein extract was quantified using the BCA protein assay reagent (PIERCE, Thermo Fisher Scientific; Rockford, IL USA) and the same amount of protein was loaded in an SDS-acrylamide gel for protein separation. Benchmarker (Invitrogen, Life Technologies) protein marker was used for protein weight reference.

After gel was resolved, proteins were transferred to a nitril or PVDF membrane during 2 hours at 100V. Membrane was blocked for non-specific interactions with 5% milk in TBS-0.5% Tween for 30 minutes. After that, primary antibody was incubated over-night at 4º in constant agitation. The primary antibody was rinsed for 30 minutes (3 washes of 10 minutes) with TBS-Tween. Then membrane was incubated with the secondary HRP-conjugated antibody for 1 hour at room temperature in constant agitation. Membrane was rinsed again with TBS-Tween and was developed with ECL (Millipore or West Dura super-signal when protein amount was low).

Table 2: List of antibodies used for Immunoblotting

Antigen	Company / Cat. no	Molecular Weight	Source
AML1	Cell Signaling (4334)	48 KDa	Rabbit
AML1	Active Motif (3900)	48 KDa	Rabbit
AKT	Cell Signaling (9272)	60 KDa	Rabbit
Cleaved Caspase 3	Cell Signaling (9661)	20 KDa	Rabbit
Cleaved PARP	Cell Signaling (9541)	89 KDa	Rabbit
CBFA2T3 (ETO2)	Abcam (Ab33072)	70 KDa	Rabbit
Firefly luciferase	Abcam (Ab64564)	62 KDa	Mouse
FLAG	Sigma (F3165)	-	Mouse
GAPDH	Trevigen (2275-PC-100)	35,8 KDa	Rabbit
GFP	Abcam (ab6556)	27 KDa	Rabbit
HA	Sigma (H9658)	-	Mouse
CD44std	Millipore (217604)	80 KDa	Rabbit
ID1	Santa Cruz (sc-488)	21 KDa	Rabbit
ID2	Santa Cruz (sc-489)	22 KDa	Rabbit
ID3	Santa Cruz (sc-490)	15 KDa	Rabbit
Lamin A/C	Santa Cruz (sc-6215)	62 KDa	Goat
p42/44 (ERK1/2)	Cell Signaling (9102)	42/44 KDa	Rabbit
p-Histone H2A.X	Millipore (05-636)	15 KDa	Mouse
p-p42/44 (p-Erk1/2)	Cell Signaling (9101)	42/44 KDa	Rabbit
p-Smad1/5/8	Cell Signaling (9511)	52-56 KDa	Rabbit

p-Smad2	Millipore (AB3849)	55-60 KDa	Rabbit
p-STAT3 (Y705)	Cell Signaling (9131)	88-92 KDa	Rabbit
Runx1	Abcam (ab23980)	50 KDa	Rabbit
Smad2	Cell Signaling (3103)	55-60 KDa	Mouse
STAT3	Cell Signaling (9132)	88-92 KDa	Rabbit
Tubulin	Sigma (T9026)	50 KDa	Mouse
Actin – HRP conjugated	Abcam (Ab49900)	42 KDa	-
Secondary anti-rabbit HRP-conjugated	GE Healthcare (NA940V)	-	Donkey
Secondary anti-mouse HRP-conjugated	GE Healthcare (NA931V)	-	Sheep
Secondary anti-goat HRP-conjugated	Jakson Immunolabs (305-035-003)	-	Rabbit

Immunoprecipitation

Cells were lysed using ELB buffer or RIPA, supplemented with protease inhibitor cocktail tablets (Roche Pharma, Schweiz, Switzerland) on ice. Proteins were quantified and 500µg-1000µg of protein were used per in each IP, in a final volume of 500µL. Primary antibody was added for an O/N IP at 4º with gentle rocking. Protein A/G sepharose beads (Santa Cruz Biotechnology) were added and incubated for 2 hours at 4º with gentle rocking. Immunocomplexes were precipitated with a spin and washed with RIPA buffer twice, and then resuspended in protein loading buffer and loaded into an acrylamide-SDS gel for resolving. Appropiate whole cell lysates were loaded in parallel as a control.

1.10. CHROMATIN IMMUNOPRECIPITATION

$9x10^6$ cells were plated for each condition and treated if necessary for 1 hour with TGFβ. Formaldehyde was added to cross-link proteins and DNA for 10 minutes at RT with gentle rocking. Cross-linking reaction was stopped by addition of Glycine 1,25M. Cells were lysed following manufacturer's instructions (Active Motif, Carlsbad, CA, USA). Briefly, cross-linked chromatin-protein complexes were shredded using sonication with MISONIX3000 Ultrasonic Cell Disruptor from Cole Palmer (Thermo Fisher) following the cycle:

- 5 seconds, output 4
- 1 second, output 0

  Total time = 4 minutes

Samples were kept cold in ice during all the process. In a small aliquot, cross-linking was reversed and DNA purified using QIAGEN purification columns to ensure the size of the DNA fragments was correct (between 200bp and 800bp). Cross-linked chromatin-protein complexes were incubated with primary antibody O/N at 4º under gentle rocking and then protein A/G magnetic beads were added. Corresponding IgG was used as a negative control and antibody anti-Acetylated Histone 3 was used as a positive control of ChIP. Imunoprecipitated complexes were washed with different astringent buffer according to manufacturer's instructions and using a magnetic rack to capture the magnetic beads. After that, cross-linking was reversed by treatment with Proteinase K and chromatin was purified. Regular PCR or quantitative RT-PCR was

performed with specific primers designed to amplify the promoter region of the genes.

Primers for amplification of LIF promoter **proximal** region were the following:

CHIP LIFprom-F: 5'-ACAAGCCAGGCAGGAAAAC-3'

CHIP LIFprom-R: 5'-GAGGGTGGGGAGAACAGAC-3'

Primers for amplification of LIF promoter **distal** region (+3000bp) were the following:

CHIP LIFdistal- F: 5'-AAGCTTCGGGACAAGCCAGGC-3'

CHIP LIFdistal- R: 5'-AAGCTTAGGAAACCTCAGATGCC-3'

GAPDH promoter primers included in the ChIP kit were used as a negative control.

1.11. SECRETED PROTEIN DETECTION: ELISA

We performed ELISA to quantify the amount of protein secreted to media. We used commercially available kits for LIF, TGFβ1 and TGFβ2 (R&D Systems, Mineapolis, MN, USA).

10^5 cells were cultured for 48-72 hours to have enough protein accumulated in the media. Media was collected and concentrated using centricone tubes (Millipore) by centrifugation at 3000g for 20 minutes. Concentrated media was incubated for 2 hours at room temperature in a 96 multiwell containing specific antibody

according to manufacturer's instructions. Colorimetric reaction was measured at 450nm wave-length and corrected for background at 560nm.

1.12. LUCIFERASE REPORTER ASSAYS

A172 or 293T cells were transfected by the lipofectamine method or calcium phosphate method with the reporter vector pGL2-basic and the corresponding promoter constructs, described in 1.1.

CONSTRUCTS

LIF promoter wild type (300 bp)

LIF promoter SBE mutant

LIF promoter Runx1 binding site mutant

SBE (Positive control)

pCMV-flag Runx1

ETO2 wild-type and mutants (R74Q and A141V)

Up to 5μg of reporter vector were transfected together with 0.5μg of Renilla-TK luciferase (as a control of transfection). All transfection were made in triplicates.

After an over-night (16 hours) transfection, cells were rinsed with PBS and incubated with fresh complete media.

Cells were treated for 20 hours with TGFβ or 3 days with Doxycicline in the case of TET-On vectors.

After that, cells were rinsed with PBS and lysed with Passive Lysis Buffer (Promega). Cells were frozen and de-frozen and pipeted thoroughly to enhance lysis.

40µL of lysed cells were mixed with luciferin (Promega) and luminescence was measured using FB12 Sirius (Berthold Detection Systems, Germany). Stop/Glo reagent was added to measure Renilla Luciferase activity and normalize the values.

1.13. FLOW CYTOMETRY AND FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

CD44 staining

105 cells were seeded and treated for the appropriate period of time.

Cells were collected, rinsed with PBS and blocked with an IgG blocking solution for 10 minutes at 4º. Anti-CD44 conjugated antibody BD Biosciences, San Jose, CA, USA) was added as it follows:

i. FITC-conjugated anti-CD44: diluted 1/25
ii. PE-conjugated anti-CD44: diluted 1/50
iii. APC-conjugated anti-CD44: diluted 1/10

Cells were incubated with the antibody for 20 minutes at 4º. After, cells were rinsed with PBS and resuspended in an Propidium Iodade (PI) containing solution (1µg/mL). Cells were immediately assessed by flow cytometry using FACSCalibur (BD Biosciences, San Jose, CA, USA) and CellQuest Pro Software (BD Biosciences).

CD44 high/low sorting

Cells were collected and rinsed with PBS. Cells were incubated for 30 minutes with a PE-conjugated anti-CD44 antibody at 4º under constant agitation.

Cells were rinsed with PBS, resuspended in 3mL of Neurobasal medium and filtered through a 30µm filter. Cells were sorted on $CD44^{high}$ or $CD44^{low}$ expressing cells using Moflo Cell Sorter (Beckman Coulter, Brea, CA, USA) and collected in polystyrene tubes. Cells were afterwards lysed for DNA, RNA or protein extraction, orthotopically inoculated in mice or maintained in culture for further experiments.

1.14. PROLIFERATION ASSAY AND SELF-RENEWAL ASSAY

Proliferation assay

5000 cells were seeded in 24 multi-well plates in replicates. Treatments were added if necessary. Cells were counted the initial day as a normalization value. Then, cells were counted at 3, 5, 7 and 10 days and proliferation curves were made. Propidium Iodide (PI) was used to exclude dead cells.

Self-renewal assay

400 disaggregated cells were seeded in a 96 multi-well plate in triplicates. Treatments were added if necessary. After 7 and 10 days, neurospheres with more than 10 cells were counted under a microscope.

In some experiments, cells were treated for a week before seeding the self-renewal assay, to assess the effect of the treatment in the proportion of neursophere initating capacity of the cells.

1.15. CELL CYCLE ANALYSIS: BROMO-DEOXI-URIDINE (BRDU) INCORPORATION ASSAY

10^4 cells were seeded in 12 multi-well plates in duplicates. Cells were treated if necessary for 5 and 10 days. Bromo-deoxi-Uridine (BrdU, 10µM) was added in each well for 8 hours. Cells were collected, washed and fixed by adding ethanol drop wise, and incubated over-night at 4º protected from light. Cells were then washed with PBS 0.5%BSA buffer and denatured with HCl (2N). Cells were incubated with a FITC-conjugated anti-BrdU antibody for 30 minutes at room temperature and then collected with a buffer containing RNAseA (100µg/mL), Propidum Iodide (5µg/mL) and TritonX-100 (0.1%). BrdU incorporation was monitored in FACSCalibur using CellQuest Pro software.

1.16. APOPTOSIS AND CELL DEATH ANALYSIS: ANNEXIN V AND SUBG1 ANALYSIS

Annexin V assay

Early and late apoptotic cells were assessed by Annexin V staining. 10^4 cells were seeded in 12 multi-well plates in duplicates. Cells were treated if necessary during 72 hours. Cells were collected and incubated with anti-annexin V APC-conjugated antibody (BD

Biosciences) for 15 minutes at room temperature and protected from light. After that, cells were resuspended in PI containing buffer (at 1µg/mL)

Analysis of SubG1 cells

Cells were collected and centrifuged for 2 minutes at 3500rpm at 4º, and washed with PBS. After that, cells were fixed by adding 70% ethanol drop wise and incubated for 30 minutes at 4º protected from light. Cells were then washed with PBS and resuspended in DNA extraction solution (Na_2HPO_4 0.2M, Citric Acid 0.1M, pH 7.8) and incubated for 10 minutes at 37º. Cells were centrifuged, washed with PBS and resuspended in a buffer containing IP (40µg/mL) and RNAse (100µg/mL) and incubated for 30 minutes at 37º. Cells were assessed using FACSAria cytometer and CellQuest Pro software (BD Biosciences).

1.17. IMMUNOFLURESCENCE OF CELLS

Cells were seeded in Collagen or Laminin pre-coated coverslides and treated if necessary. Cells were rinsed and fixed with 4% paraformaldheide freshly prepared during 30 minutes at room temperature. Then cells were permeabilized with PBS-Triton X-100 (0.1%) for 20 minutes at room temperature. Unspecific interactions were blocked by incubating cells with PBS 5% BSA during 1 hour. After that, primary antibody was added at the cover-slides and incubated at 4º over-night (16 hours). Next day, primary antibody was washed with PBS and secondary fluorescent antibody was added (Alexa Fluor 488 and 594, Invitrogen, Life Technologies) together with Hoechst 33258 for nuclear staining

(Sigma) for 1 hour at room temperature. Cells were washed carefully with PBS and then coverslides were mounted with Fluoromount-G into glass slides.

Immunofluorescence samples were immediately assessed under fluorescence microscope or stored at 4 or -20º protected from light.

2. *IN VIVO* TECHNIQUES

2.1. GLIOBLASTOMA XENOGRAFT MOUSE MODEL

10^5 cells were collected and rinsed with PBS, centrifµged at 400g for 5 minutes and resuspended in 5µL of PBS. Cells were kept in ice to avoid cell death.

NOD-SCID immunocompromized mice were anesthetized with intraperitoneal administration of Ketamine/Xylacine (75mg/Kg and 10mg/Kg). Each mouse was carefully situated in the stereotactic and immobilized. Hair from head was removed with depilatory cream; the head skin was cut with a scalpel and to expose the skull. A small incision was performed carefully with a drill the coordinates 0.8mm lateral / 1mm anterior from Bregma. Cells were inoculated using a Hammilton 30G syringe directly at the brain of the mouse, at 2.5mm of depth (in the right striatum). Head incision was closed with Hystoacryl tissue adhesive (BRAUN, Mesulgen, Germany) and mice were injected with subcutaneous analgesic Meoxicam (1mg/Kg).

Depending on the cells inoculated, tumors took from 2 to 6 months to develop. Tumors recapitulate the characteristics of the patient tumor, making this model very useful for pre-clinical studies (see figure 1.11).

2.2. *IN VIVO* TREATMENTS

In vivo treatment with TβRI inhibitor

We orally treated mice with a TGFβ inhibitor (LY2109761 and LYLY 2157299) at 75mg/Kg, twice a day. The control (Placebo) group

was treated only with the vehicle (NaCMC 1%, SLS 0.5%, Antifoam 0.05%)

For TET-On experiments, doxycicline mixed with sucrose was added at the water. Placebo animals have sucrose added in the water.

In vivo irradiation of mice

Mice were anesthetized with an intra-peritoneal injection of Ketamine/Xylacine (75mg/Kg and 10mg/Kg) and heads were carefully placed in a Cobalt radioactive source. Mice were irradiated at a single dose of 9Gy which is equivalent to what patients receive when they are under radiotherapy and shown no significant toxicity for the animals.

2.3. MRI QUANTIFICATION OF TUMOR AREA

Magnetic resonance imaging (MRI) analysis was performed on mice injected intraperitoneally with gadolinium diethylenetriamine penta-acetic acid at a dose of 0.25 mmol gadolium/kg body weight. T1 W magnetic resonance images were acquired in a 9.4 T vertical bore magnet interfaced to an AVANCE 400 system (Bruker) using a spin-echo sequence as described previously.

2.4. *IN VIVO* QUANTIFICATION OF LUCIFERASE ACTIVITY

Cells were stably infected with constitutively active luciferase vectors (purchased from Addgene) (Figure 2.7). We used 2 different vectors, one having the Luciferase gene under a pCMV

constitutive promoter and harboring Puromycin resistance and another one with a PGK constitutive promoter controlling Luciferase expression and with Neomycin (G-418) resistance.

Luciferase activity was assessed *in vitro* prior to inoculation of the cells in mouse using FB12 Sirius.

Luciferase activity was quantified using IVIS Spectrum (IVISSPE, Perkin Elmer; Waltham, MA, USA) *in vivo* imaging system. Mice were anesthesyzed using isofluorane and injected intraperitoneally with luciferin substrate. Luciferase intensity was measured and correlated with tumor size. Tumor growth and response to treatment was monitorized every week.

2.4. IMMUNOFLUORESCENCE AND IMMUNOHISTOLOGICAL TECHNIQUES

Mice brains were surgically removed and frozen with Isopentane and dry ice and kept at -80ºC for long term storage.

Frozen brains were cut in 10µM slices in the cryostat. Positively charged coverslides from DAKO (Glostrup, Denmark) were used. Sections were immediately fixed in methanol:acetic acid (3 to 1 proportion) or frozen at -80 for long term storage.

Slides were treated with 0.5% trypsin for 5 minutes at room temperature and permeabilized with PBS-1% Tween for 20 minutes at room temperature. Unspecific interactions were blocked with 10% FBS for 30 minutes at 37º.

Primary antibody was added at the right concentration with 3%BSA on the top of the section in a humid chamber and incubated over-night (16 hours) at 4º in a still position.

For immunofluorescence, slides were washed with PBS for 30 minutes (3 washes of 10 minutes) and secondary fluorescence antibody was added diluted at 1:200 in 3%BSA in PBS. Hoechst (Bis-benzimide Hoechst-33258, SIGMA) was added at the same mixture. Slides were incubated for 1 hour at 37º in a humid chamber protected from light. Afterwards slides were washed with PBS for 30 minutes protected from light and mounted with Flouromount-G. Immunofluorescence were immediately assessed in the fluorescence microscope or stored at 4/-20º for long term storage, protected from light.

For immunohistochemistry, the secondary antibody used was ENVISION plus (DAKO) a mixture of anti-mouse/rabbit secondary antibodies HRP-conjµgated. The antibody was added on the top of the slides and incubated at room temperature for 20 minutres in a humid chamber protected from light. Then slides were rinsed with TBS-0.5% Tween for 30 minutes (3 washes of 10 minutes). HRP was developed using Diaminobenzidine (DAB) freshly prepared (DAKO). DAB was added to the slides and staining was carefully monitorized under a light microscope to ensure the correct development of the signal. Then slides were counterstained with haematoxilin harris (LEICA- SIGMA) for 20 seconds and rinsed under current water for 2 minutes. Samples were dehydrated in a serie of ethanols (70% --90% --100%) and 3 clean xylene solutions. Samples were mounted using DPX mounting medium (VWR) and air dry before storage or viewing in a light microscope.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

For assessing Apoptosis in tumor samples we used *In situ* Cell Death Detection Kit TMR-Red from Roche, which detects DNA double-strand breaks that are typical from apoptotic cells. Following manufacturer's instructions samples were fixed in 4% Paraformaldheide solution in PBS freshly prepared for 20 minutes. After washing with PBS, slides were permeabilized for 2 minutes and washed again in PBS. Then samples were incubated with Label Solution for 1 hour at 37º. Hoechst was added during 15 minutes as a nuclei counterstaining. DNAseI treated sample was used as a positive control. Samples were mounted with Fluoromount-G (Southern Biotech, Birmingham, AL, USA) and protected from light.

2.5. SORTING OF HUMAN CELLS

Mice brains were surgically removed and dissected in two parts. Each part was carefully chopped with a scalpel and digested with DNAseI (SIGMA) and Collagenase (SIGMA) for 1 hour at 37º under agitation. Cells were filtered through a 70µm strainer and washed with abundant PBS and centrifuged for 10 minutes at 500g. All pelleted cells were resuspended in 15mL of PBS with 115µL anti HLA classI antibody (Santa Cruz) and incubated at 4º for 30 minutes in constant agitation. Then cells were washed with PBS and centrifuged for 10 minutes at 500g. Pelleted cells were resuspended in 10mL of PBS with secondary anti-mouse RPE-conjugated antibody (DAKO) during 30 minutes at 4º in constant agitation. Cells were washed with PBS and centrifuged for 10 minutes at 500g. Cells were resuspended in 3mL of complete

Neurobasal medium (GIBCO) and filtered before sorting. Positive cells for MHC ClassI (HLA I) were sorted with MoFlo Cell sorter and collected in polyestirene round tubes (BD Falcon). Cells were immediately lysed for RNA/protein extraction, cultured in neurobasal medium or re-inoculated in NOD/SCID mice.

2.7. STATICAL ANALYSIS

Kaplan-Meyer survival curves

Graph-Pad Prism 5.0 software [http://www.graphpad.com/] was used for generation of Kaplan-Meier survival curves showing the probability of survival over time. Mice were euthanized when they presented neurological symptoms or significant weight loss, and we counted days of survival for each mouse.

Statistics were calculated using Graph-Pad. P value was calculated for each group.

Student T test and ANOVA analysis of variation

To compare two different groups we used Student's T test (paired or unpaired) for parametric variables and Mann-Whitney test for non-parametric variables. To compare different groups of samples, we used One-Way ANOVA test for parametric variables, coupled with a Bonferroni post-test or Kruskal-Wallis test for non-parametric variables.

3. PATIENT TISSUE SAMPLES

3.2. IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE IN FORMALIN-FIXED PARAFFIN-EMBEDDED (FFPE) TUMOR SAMPLES

Immunohistochemistry in formalin-fixed paraffin-embedded (FFPE) tumor samples

Tumor samples were fixed in formol over-night (16 hours) and then included in paraffin for long term storage. When necessary, samples were cut at 5µM in a microtome and tumor slices were placed in DAKO+ charged slides.

Slides were heated at 65º for 3 hours to over-night and deparafinized in a serie of 3 xylenes. Slides were hydrated in a serie of decreasing ethanols (100% -- 90% --70%) and distilled water. Heat mediated antigen retrival (HIER) was performed in a histoprocessor.

- Citrate Buffer pH6 (DAKO) – 115º , 5 minutes
- Buffer pH9 (DAKO) – 110º, 5 minutes
- Citrate Buffer pH 7.3 (home made) – 110º
- EDTA Buffer pH8 – 110º, 4 minutes

The conditions were set up for each antibody using appropriate positive controls.

Peroxidase was blocked with a 3% H_2O_2 solution for 10 minutes protected from light. Slides were rinsed with TBS-Tween 10% (3 washes of 5 minutes). Samples were incubated with blocking solution (2%BSA and 10% normal goat serum – Invitrogen in TBS-Tween) for 30

minutes or 1 hour 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 (DAKO) at 4º over-night in a humid chamber in a still position.

The next day, primary antibody was rinsed in 3 washes with TBS-Tween and ENVISION secondary HRP-conjugated antibody (mouse/rabbit) was added on the top of slides and incubated for 20 minutes at room temperature in a humid chamber. Slides were then rinsed in 3 washes with TBS-T and developed with diaminobenzidine (DAB) under a light microscope to ensure the correct development of the signal. Then slides were counterstained with Harris haematoxilin (LEICA- SIGMA) for 20 seconds and rinsed under current water for 2 minutes. Samples were dehydrated in a serie of ethanols (70% -- 90% --100%) and 3 clean xylene solutions. Samples were mounted using DPX mounting medium (VWR) and air dried before storage or viewing in a light microscope.

Table 3: List of primary antibodies used for IHC

Antigen	Company /Cat no	Source	Dilution	HIER
LIF	R&D (AF-250-NA)	Goat	1:20	pH 9
LIF	Atlas Antibodies (HPA018844)	Rabbit	1:100	pH 6
Phospho-Histone H2A.X(Ser 139)	Millipore (05-636)	Mouse	1:100	pH 6
Cleaved Caspase-3 (Asp175)	Cell Signaling (9661)	Rabbit	1:500	pH 6
Id1	Biocheck (BCH-1/195-14)	Rabbit	1:100	pH 6
Id3	Biocheck (BCH-4/17-3)	Rabbit	1:100	pH 6
CBFA2T3 (ETO2)	Abcam (ab110823)	Rabbit	1:100	pH 6
CD31 (PECAM1)	Invitrogen (Clone	Mouse	1:50	pH 8

	1A10)			
CD31	DAKO (Clone JC70A)	Mouse	1:50	pH6
Endoglin	R&D systems	Goat	1:50	pH6
Ki-67	DAKO (Clone MIB-1)	Mouse	1:100	pH 6
CD44std/HCAM Ab-4	Thermo Scientific (clone 156-3C11)	Mouse	1:100	pH 6
CD44std	Bender MedSystems (BMS113)	Mouse	1:100	pH 6
Runx1	Atlas Antibodies (HPA004176)	Rabbit	1:50	pH 6
Nestin	Millipore (MAB5326)	Mouse	1:200	pH 9
Phospho-STAT3 (Tyr705)	Cell Signaling (9131)	Rabbit	1:100	pH 6
PDGFA	Santa Cruz (sc-9974)	Mouse	1:50	pH 9
PDGF-Receptor alpha	Abcam (ab118514)	Rabbit	1:200	pH 6
B-Catenin	BD (610154)	Mouse	1:500	pH 6
Phospho-Smad2	Cell Signaling (3108)	Rabbit	1:150	pH 6
TGFb2	Santa Cruz (sc-90)	Rabbit	1:200	pH 6
YKL-40	QUIDEL (4185)	Rabbit	1:100	pH 6
Olig-2	IBL (18953)	Rabbit	1:200	pH 6
Met	Santa Cruz (sc-8057)	Mouse	1:100	pH 6

Tissue Microarrays

Selected areas from human 43 GBM samples were chosen by an expert pathologist and were spoted into 4 tissue microarrays we generated. We have 3 GBM tissue microarrays and one tissue microarray from low grade gliomas. For each patient, 3 representative spots were selected. We processed each slide from TMA as a slide from FFPE sample for

immunohistochemistry. After that, a pathologist evaluated the intensity of the staining and calculated the **H-Score** according to formula:

3 x percentage of strongly staining cells + 2 x percentage of moderately staining cells + percentage of weakly staining cells, giving a range of 0 to 300 (Ishibashi, Suzuki et al. 2003).

Immunofluorescence in formalin-fixed paraffin-embedded samples

Similary to immunohistochemistry in FFPE samples, tumor samples were fixed in formol over-night (16 hours) and then included in paraffin. 5µm Slides were heated at 65º for 3 hours to over-night and deparafinized in a serie of 3 xylenes. Slides were hydrated in a serie of decreasing ethanols (100% -- 90% --70%) and distilled water. Heat mediated antigen retrival (HIER) was performed in a histoprocessor, using pH6 citrate buffer, at 115º for 5 minutes. After cooling down the samples, they were permeabilized using 1 to 2% solution of PBS-Tween for 20 minutes. Samples were incubated with blocking solution (2%BSA in TBS-Tween) for 30 minutes or 1 hour at room temperature in a humid chamber. Then slides were rinsed with TBS-Tween and incubated with the primary antibody diluted in 3% BSA at 4º over-night in a humid chamber in a still position. The following day, primary antibody was rinsed in 3 washes with TBS-Tween and fluorescence-labeled secondary antibody (anti-mouse or rabbit) was incubated during 1 hour at room temperature in a humid chamber. Typically, Alexa Fluor 594 and 488 secondary antibodies were used diluted 1:200

in 3% BSA (Invitrogen, Life Technologies). Hoechst (Bis-benzimide Hoechst-33258, SIGMA) was added to the mixture to counterstain nuceli. Slides were then rinsed in 3 washes with PBS and analyzed under fluorescence microscope or stored at -20º for long-term storage.

3.4. CONFOCAL AND IMAGE J ANALYSIS

Immunofluorescence slides were visualized using Olympus FluoView FV1000 Confocal microscope with its software.

ImageJ software was used for fluorescence quantification or cell counting.

3.5. ANALYSIS OF PATIENT-SAMPLE DATABASES

Oncomine

Oncomine database [www.oncomine.org] is a cancer microarray database aimed to facilitate the study and data-mining of genome-wide expression analyses. It contains gene expression measurements from nearly 5,000 micrarray experiments (Rhodes, Yu et al. 2004). In this case, we used it to compare gene expression between tumor and healthy tissue and between different tumor subtypes or grades.

GeneSapiens

GeneSapiens [www.genesapiens.org] is a database useful to compare gene expression levels among different healthy or pathologic tissue samples. It can also run correlations between different genes in a certain sample dataset (Kilpinen, Autio et al. 2008). In this case, it was

used to correlate the levels of expression between the genes *RUNX1* and LIF in glioma samples.

REMBRANDT

Repository for Molecular BRAin Neoplasia DaTa (REMBRANDT) is a bioinformatic framework that integrates clinical and functional genomics as well as data from clinical trials of glioma patients (Madhavan, Zenklusen et al. 2009). With this tool, we can asses gene expression, chromosome aberrations and how they affect clinical data such as overall survival.

3.5 STATISTICAL ANALYSIS

To calculate the statistical significance and correlation between different genes in patient samples, we used Pearson correlation for parametric variables following a Gaussian distribution, and we calculated the p value and Rsquared coefficient of correlation. For Non-parametric variables, we used Spearman test and calculated p value and Spearman rho value.

4. *IN SILICO* TECHNIQUES

ANALYSIS OF LIF PROMOTER REGION TO SEARCH FOR TRANSCRIPTION FACTOR BINDING SITES

Promoter region from human *LIF* gene and orthologs were found using UCSC Genome Browser website [http://genome.ucsc.edu/cgi-bin/hgGateway]. Genomic DNA sequence was obtained and -1000 bp promoter region was used for posterior analyses. Different ortholog promoter regions were aligned using ClustalW [http://embnet.vital-it.ch/software/ClustalW.html]. Evolutionary conserved regions were analyzed using ECRGenome Browser [http://ecrbrowser.dcode.org/] . Selected regions were used for MEME analysis of conserved motifs [http://meme.nbcr.net/meme/doc/overview.html] and motifs were analyzed for Transcription Factor Binding Sites (TFBSs) using JASPAR database of TFBS matrices [http://jaspar.genereg.net/]. TRANSFAC [http://www.gene-regulation.com/cgi-bin/pub/databases/transfac/search.cgi] and TFSearch [http://www.cbrc.jp/research/db/TFSEARCH.html] were also used to validate the results and find putative TFBSs. These putative TFBSs were validated using *in vitro* experiments.

RESULTS

1. TGFβ PATHWAY ACTIVITY IS IMPORTANT FOR GICS CD44 ^{HIGH} /ID1 POSITIVE IN GBM

As discussed earlier in the introduction chapter, the TGFβ pathway has been reported to have an important oncogenic role in cancer. Moreover, we and others have described that TGFβ activity is crucial for Glioma Initiating Cells (Ikushima, Todo et al. 2009; Penuelas, Anido et al. 2009; Seoane 2009). We are interested in the study of GIC population as they are responsible for tumor initiation, resistance and recurrence. The study of the molecular mechanisms and pathways that regulate this entity would improve the therapy for GBM patients. For this reason, inhibitors that specifically target the TGFβ pathway have been developed and are currently entering into clinical trials. (Arteaga 2006; Seoane 2008).

TGFβ INHIBITION GENE RESPONSE INCLUDES DOWN-REGULATION OF ID1 AND ID3

We were interested in understanding the molecular mechanism of the oncogenic effect of the TGFβ pathway inhibition in glioma and especially focusing in GICs. In order to study the response to the TGFβ inhibitor, we treated 11 patient-derived cell cultures with the highly selective TβRI inhibitor LY2109761 from Eli-Lilly (Figure 3.1 A) and we performed microarray gene-expression analysis. Among the transcripts that were modulated by the TβRI inhibitor we focused our interest on Inhibitors of Differentiation 1 and 3 (Id1 and Id3) (Figure 3.1 B and C) which were the most significantly down-regulated genes by the TGFβ inhibitor. Id1/3 are described to regulate cell cycle and differentiation and have an important

role in the control of stem cell self-renewal (Ruzinova and Benezra 2003; Perk, Iavarone et al. 2005; Gupta, Perk et al. 2007). Recently, Id1 has been shown to be expressed in B1 type adult neural stem cells, having an important role in the regulation of self-renewal capacity of these cells (Nam and Benezra 2009). In cancer, Id1 is found up-regulated in several tumors and has been described to have a role in metastasis (Perk, Iavarone et al. 2005; Gupta, Perk et al. 2007). We validated *ID1* and *ID3* down-regulation by treating different patient-derived neurosphere cultures with TβRI inhibitor *in vitro*. We observed a significant and reproducible decrease in *ID1* and *ID3* mRNA expression in all the cases that we have studied, upon treatment with the TβRI inhibitor (Figure 3.1 D).

We then wanted to address if Id1 and Id3 were modulated by *in vivo* treatment with the TβRI inhibitor. Mice were inoculated with patient-derived neurospheres and treated twice a day for 30 days with the TβRI inhibitor. We analyzed expression of Id1 by immunohistochemistry and immunofluorescence. Because endothelial cells are also positive for Id1, we performed co-immunofluorescence of CD31 endothelial marker and Id1, and observed that Id1 was expressed in tumor cells (Figure 3.2 A) and we observed a significant decrease in Id1 expression in tumor cells after the treatment with the TβRI inhibitor (Figure 3.2 B and C). We also observed a reduction in tumor area when monitored by MRI (Figure 3.2 E and F) and increased survival of mice after treatment with the TβRI inhibitor (Figure 3.2 G).

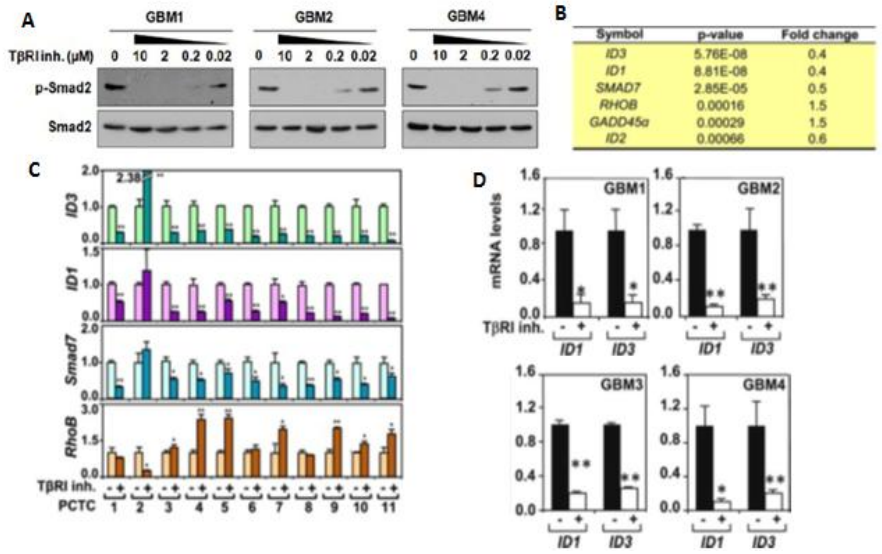


Figure 3.1. TβRI inhibition includes Id1 and Id3. **A.** Western blott analysis of p-Smad2 in four different patient-derived cultures. At 2 μM, TβRI inhibitor was able to completely block phosphorylation of Smad2 and TGFβ pathway activation. **B.** The 6-gene signature of TGFβ inhibition was obtained by microarray gene expression analysis. The genes regulated by the treatment with TβRI inhibitor for 3 hours in 11 human Glioma PCTCs with a fold change over 1.4 or below 0.6 and a $p < 0.001$ **C.** Validation of the genes regulated by TβRI inhibitor by quantitative Real-Time PCR. *ID1*, *ID3*, *SMAD7* and *RHOB* transcript levels were determined by qRT-PCR analysis. *GAPDH* RNA levels were used as an internal normalization control. **D.** mRNA levels of Id1 and Id3 of 4 different GBM samples treated with TβRI inhibitor were determined by qRT-PCR. * $p< 0.05$; **$p<0.001$. Data are presented as mean $\pm$ SD.

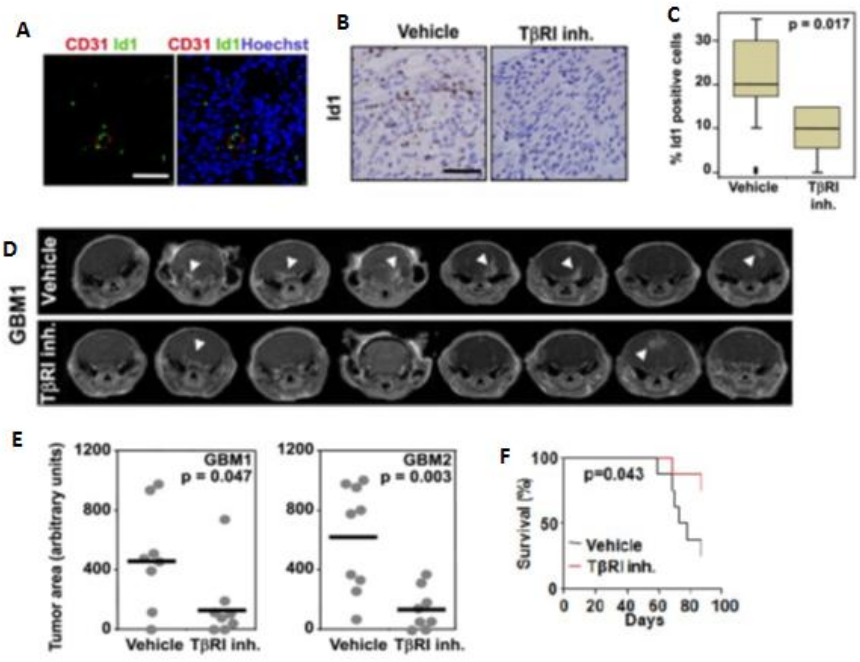


Figure 3.2. *In vivo* TβRI inhibition decreases Id1 **A**. Id1 and CD31 coimmunofluorescence was performed to show that Id1 positive cells were tumor cells. B. Id1 immunohistochemistry showing nuclear staining of ID1. Animals treated with TβRI inhibitor had lower Id1 signal when quantified by IHC (**C**). **D.** Mice harboring patient-derived tumors were treated for 40 days with TβRI inhibitor. We observed a decrease in tumor area by MRI (**D** and **E**) and increased overall survival (**F**).

CD44^{HIGH} POPULATION HAS CANCER INITIATING CAPACITY IN VIVO AND CORRELATE WITH *ID1* EXPRESSION

When we assessed the expression of Id1 protein in neurosphere cultures we observed that there was a heterogeneous pattern, with some cells expressing high levels of Id1 and others with no Id1 expression. We then performed co-staining with different described Cancer Initiating Cell markers such as CD44, CD133 and SSEA-1. Id1 expression correlates with CD44 but not with other Cancer Initiating Cell markers (Figure 3.3 A and B). We analyzed different patient-derived samples and we observed two different populations of CD44-expressing cells (Figure 3.3 C).

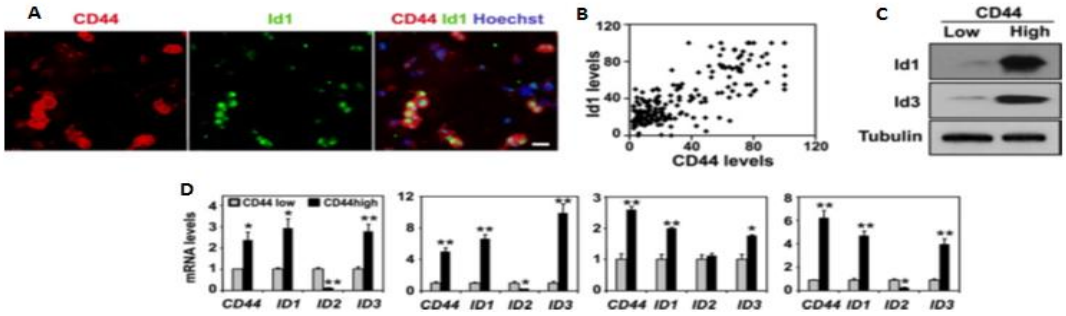


Figure 3.3. CD44 expression correlates with Id1. **A.** Co-immunofluorescence staining reveals that Id1 expression correlates with CD44 expression in patient-derived cells. Scale bar, 10 µm. **B**. Quantification of Id1 and CD44 expression levels per cell is shown. **C.** Cells from GBM1 neurospheres were sorted by FACS according to CD44 levels, and the levels of Id1, Id3, and tubulin were determined by immunoblotting. **D.** Cells from different GBM patients were sorted and levels of *ID1*, *ID2* and *ID3* were determined by qRT-PCR showing an enrichment of *ID1* and *ID3* expression in CD44^{high} population in 4 different patient-derived neurospheres. $*p < 0.01$; $**p < 0.001$. Data are presented as means $\pm$ SD.

When CD44 high and low sub-populations of four different GBM samples were sorted, we observed that Id1 and Id3 were predominantly expressed in the CD44^{high} compartment (Figure 3.3 C and D).

To address if CD44^{high} population were indeed GICs, we first assessed those cells show higher self-renewal capacity. We sorted CD44^{high} and low cells and we seeded them at low density. We observed that CD44^{high} cells generate more neurospheres, indicating that they have enhanced self-renewal capacity, which is characteristic of GICs (Figure 3.4 A). In order to address whether CD44^{high} cells have greater tumor initiating capacity, we inoculated limiting dilutions of CD44^{high} and CD44^{low} cells into immunocompromised mice. CD44^{high} cells were able to generate tumors more efficiently than CD44^{low} cells, showing that indeed they have tumor initiation capacity. (Figure 3.4 B and C).

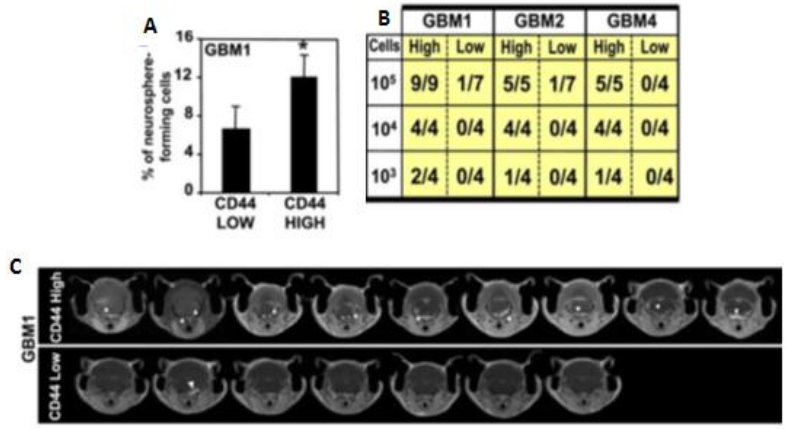


Figure 3.4. CD44^{high} cells have increased self-renewal capacity. **A.** Patient-derived neurospheres were sorted depending on the CD44 levels and seeded at low density for self-renewal assay. CD44^{high} generated more neurospheres compared to CD44^{low}. **B.** CD44high cells have increased tumor initiation capacity. CD44 high and low populations from different GBM patients were sorted and inoculated orthotopically into NOD/SCID mice. Tumors were monitored by MRI (**C**).

Taking together, our results indicate that the $CD44^{high}$ compartment is enriched for GICs, as has been shown in other tumor types.

TβRI INHIBITOR REGULATES GIC POPULATION $CD44^{HIGH}$/ID1+ *IN VITRO* AND *IN VIVO*

Keeping in mind that Id's proteins have been shown to be involved in stem cell biology (Nam and Benezra 2009) we therefore hypothesized that Id1 downregulation by the TβRI inhibitor may be relevant for the maintenance of GICs. In order to assess the effect of the TβRI inhibitor on GBM neurospheres, we dissociated them into single cells, plated at low density, treated with the TβRI inhibitor for 7 days, and then, we counted the newly formed neurospheres. Treatment of GBM neurospheres with the TβRI decreases the number of neurosphere-forming cells (Figure 3.5 A). We also treated patient-derived neurospheres with TGFβ for 7 days and an increase of the $CD44^{high}$ percentage was observed, while neurospheres treated with TβRI inhibitor showed a decrease of the $CD44^{high}$ compartment (Figure 3.5 B and C). Altogether, our results demonstrate that TGFβ regulates the $CD44^{high}$ compartment enriched for GICs and that this is a result of a transdifferentiation process.

In vivo, treatment with the TβRI inhibitor efficiently reduced tumor volume (Figure 3.2 D). We wanted to test if Id1 down-regulation was responsible for this effect. In order to address that, we orthotopically inoculated cells with *ID1*/3 knock-down and tumor progression was followed by MRI. We observed that cells with lower levels of Id1/3 generated smaller tumors and with lower incidence compared to control cells (Figure 3.6 A-C). We also pre-treated neurospheres in vitro with the TβRI inhibitor and we

observed a similar effect on tumor initiation capacity, further supporting the hipothesis that TβRI inhibitor effect may be mediated by Id1 and Id3 and also decreasing the tumorigenic capacity of patient-neurospheres (Figure 3.6 A-C).

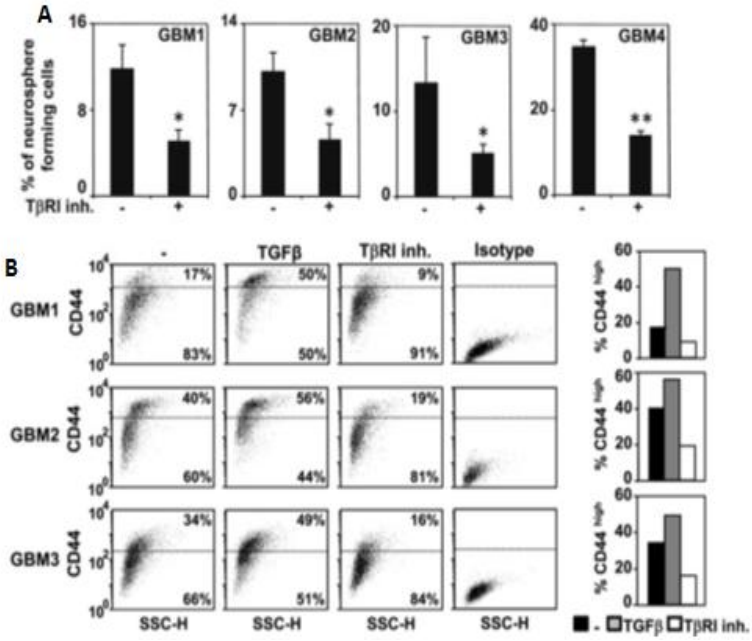


Figure 3.5. TβRI inhibitor regulates GIC population CD44^{high}/Id1+ *in vitro.* **A.** Cells from different patient-derived neurospheres were dissociated, plated at low density and treated with 2 µM TβRI inhibitor for 10 days. Number of newly formed neurospheres was couted as a readout of self-renewal capacity. **B.** Cells from the indicated GBM neurospheres were left untreated or treated with 100 pM TGFβ or 2 µM TβRI inhibitor for 10 days. CD44 levels were determined by FACS analysis. Right panels show quantification of the percentage of CD44high cells. * p< 0.05. Data are presented as mean ± SD.

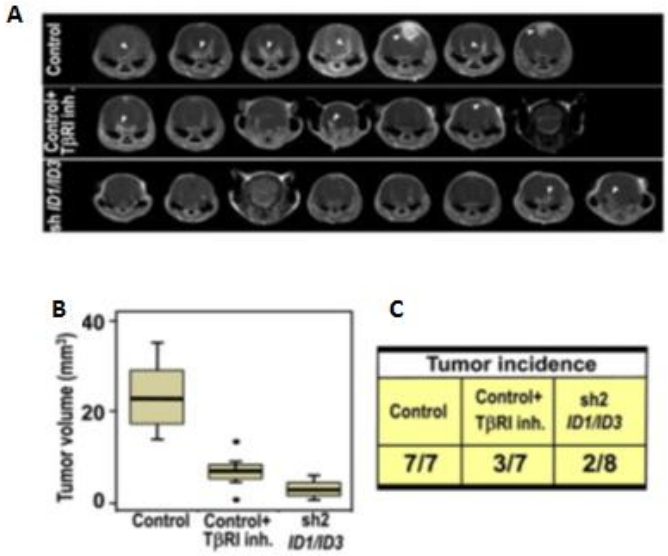


Figure 3.6. TβRI inhibitor regulates GIC population CD44^{high}/Id1+ *in vivo.* **A.** GBM1 control neurospheres and neurospheres with ID1 knock-down were treated for 7 days with 2 µM TβRI inhibitor, or left untreated. Subsequently, equal numbers of cells were inoculated in the brain of NOD-SCID mice. Images from the entire mouse brains were obtained by MRI. Arrowheads indicate tumors. **B.** Tumor area was quantified ($p = 0.004$ comparing mice inoculated with untreated neurospheres with mice inoculated with neurospheres treated with the TβRI inhibitor; $p = 0.002$ comparing mice inoculated with control neurospheres with mice inoculated with neurospheres with knock-down of *ID1/ID3*). **C**. Tumor incidence was determined. Data are presented as means ± SD.

GICS CD44^{HIGH}/*ID1*+ TEND TO BE LOCATED IN A PERIVASCULAR NICHE IN GBM PATIENTS

In order to confirm that Id1 positive and CD44 high GICs were present in glioma patient samples we performed immunohistochemical (IHC) staining of Id1 in a Tissue Microarray of 43 GBM patients. Frequency of Id1 positive nuclei was calculated (Figure 3.7 A). We also performed IHC of CD44 and Id1 in serial slides of the same tumor and co-immunofluorescence of paraffin-embedded glioma samples that were resected in our hospital. We observed some cells that express high levels of CD44 and some, but not all of them, were also positive for Id1 (Figure 3.7 B). Interestingly, those cells tend to be located in the proximity of tumor blood vessels (stained by an endothelial surface marker CD31) suggesting that they need to be in a specific microenvironment to maintain their GIC characteristics. This result is in concordance with the fact that it has been previously reported that GICs tend to be located in a perivascular niche in GBM (Calabrese, Poppleton et al. 2007). We quantified the proportion of CD44^{high}/Id1 positive cells located within 100µm of blood vessels. In four different GBM patients, the proportion of those cells near the tumor vessels was higher than cells located further from vessels (Figure 3.7 C).

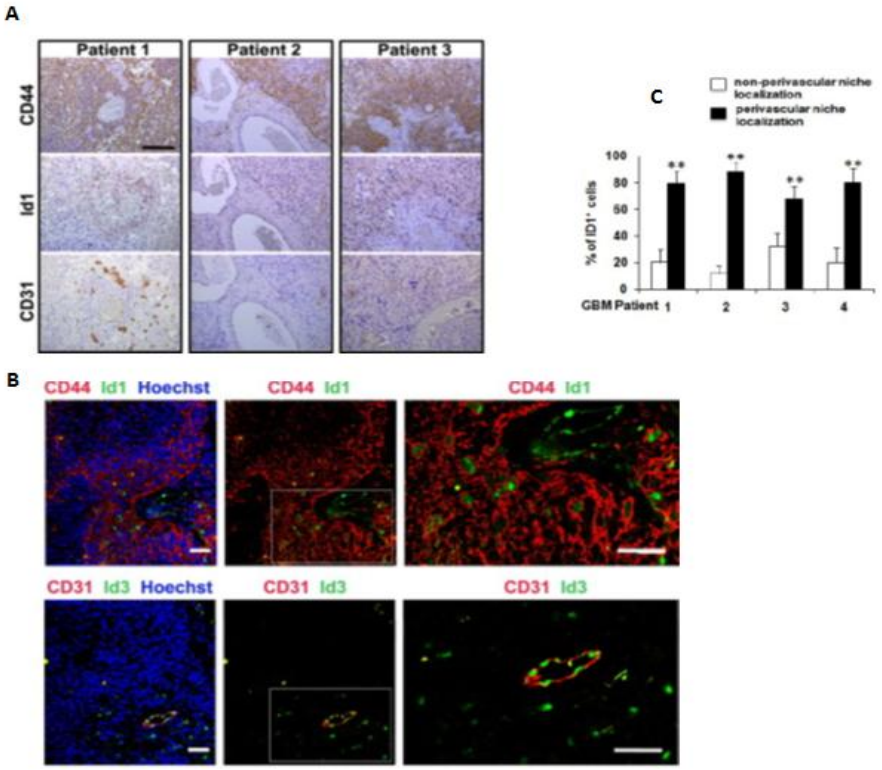


Figure 3.7. GICs CD44^{high}/Id1+tend to be located in a perivascular niche in GBM patients. **A.** Id1 and CD44 IHC was performed in paraffin-embedded GBM samples. **B.** Id1 and CD44 co-immunofluorescence was performed in paraffin-embedded GBM samples. CD31 was used as an endothelial cell marker to discard Id1 positive endothelial cells. The careful analysis of the Id1 staining showed that, in around 20% of tumors, *ID1*-expressing cells tend to localize in the proximity of tumor vessels**. C.** Five randomly selected 10x fields were quantified using ImageJ software. Id1 positive cells tend to be located proximal (< 100µm) to tumor vessels in four different GBM patients. ** = p value < 0.001. Data are presented as means ± SD.

2. ENDOTHELIAL CELLS SECRETE TGFβ CREATING A PERIVASCULAR NICHE TO MAINTAIN GIC POPULATION

CD44^{HIGH}/ID1 POSITIVE GICS ARE LOCATED IN A PERIVASCULAR NICHE WHICH HAS HIGH LEVELS OF TGFβ

We have previously reported that CD44^{high}/Id1 positive GICs tend to be located in the proximity of tumor vessels (Figure 3.7). It has been described that GICs, as well as normal stem cells, need to be located in specific niches where they receive appropriate signals from the microenvironment (such as growth factors, cytokines, etc.) that maintain their undifferentiated state. It has been reported that there is a perivascular niche in glioblastoma (Calabrese, Poppleton et al. 2007) and GICs are located in the proximity of tumor vessels, where they have the appropriate microenvironmental signals. We observed that CD44^{high}/Id1 positive GICs are indeed located in the proximity of tumor vessels in different GBM samples (Figure 3.7). Previous work from our group has shown that TGFβ is important for GIC self-renewal and LIF is one of the main mediators of this effect (Penuelas, Anido et al. 2009; Seoane 2009; Anido, Saez-Borderias et al. 2010). So we hypothesized that GIC population CD44^{high}/Id1 positive, were located in the proximity of tumor vessels because TGFβ was present in this niche, and that this TGFβ was necessary to maintain their properties such as self-renewal and tumor initiation capacity.

First of all we wanted to address if TGFβ was present in this perivascular areas colocalizing CD44^{high}/Id1 positive GICs. To do so, we performed

immunofluorescence staining of LIF and TGFβ2 (we were not able to perform immunofluorescence of TGFβ1) and we observed that both TGFβ and LIF were also located in this perivascular niche, surrounding tumor vessels, which can be identified with a CD31 endothelial marker (Figure 3.8). There was partial co-localization between CD44 and Id1 (markers of GICs) and TGFβ2 and LIF levels in four different GBM patients studied.

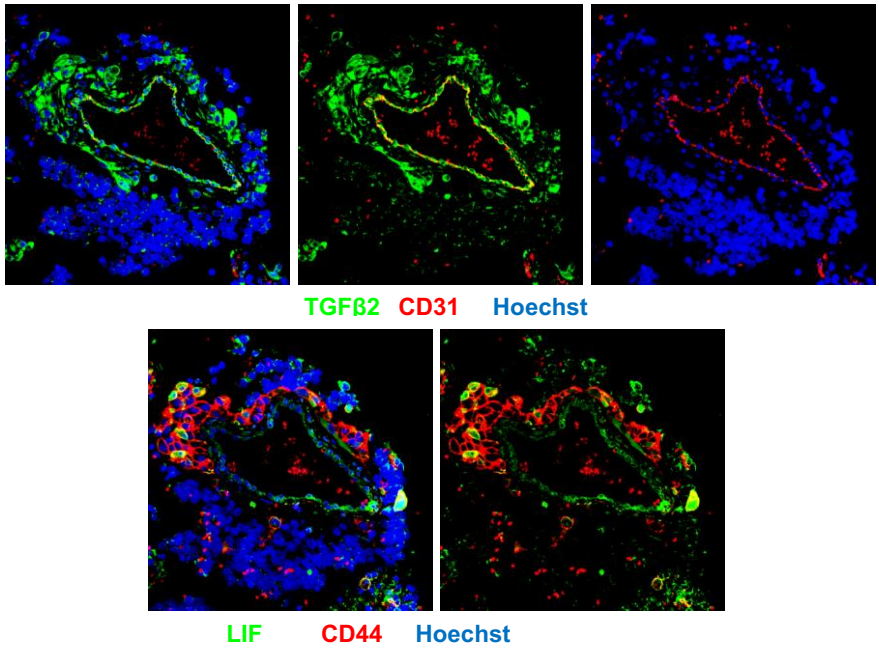


Figure 3.8. Coimmunofluorescence was performed with antibodies staining TGFβ2, LIF and CD44 (marker of GICs) and CD31 (marker of endothelial cells). Hoechst was used to counterstain nuclei. A representative section of a GBM patient is shown.

ENDOTHELIAL CELLS SECRETE TGFβ1 AND 2 AND ACTIVATE THE TGFβ PATHWAY IN PATIENT-DERIVED NEUROSPHERES

Because TGFβ was found in the proximity of blood vessels, we hypothesized that endothelial cells may be secreting TGFβ2. To test this, we cultured Human Umbilical Vein Endothelial Cells (HUVEC) cells and human Cerebral Microcapillar Endothelial Cells (hCMEC) and we analyzed by ELISA the secreted proteins found in the conditioned media (Figure 3.9 A). We observed that both endothelial cell lines secreted TGFβ1 and TGFβ2 (Figure 3.9 B). We did not observe any secretion of LIF by the endothelial cells so we postulate that the levels of LIF observed in the perivascular niche are secreted by tumor cells or others.

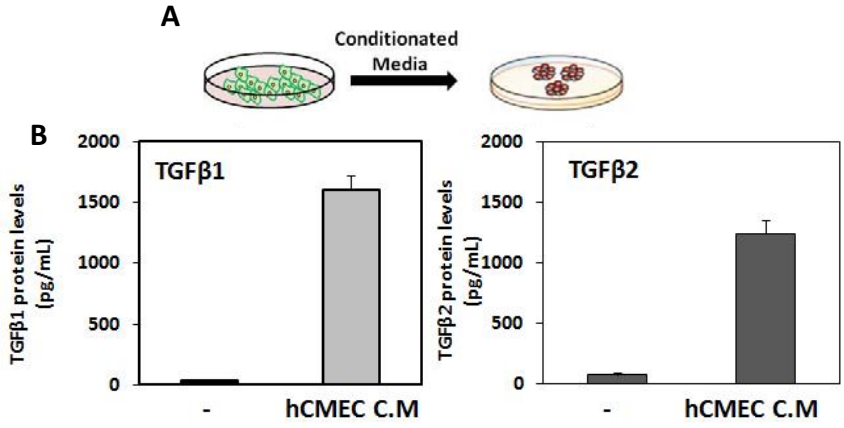


Figure 3.9. TGFβ1 and TGFβ2 are secreted by endothelial cells. **A.** Schematic representation of the procedure. Human Umbilical Vein Endothelial Cells (HUVEC) and human Cerebral Microvascular Endothelial Cells (hCMEC) were cultured in pre-coated dishes for 3 days. The conditioned media was then added to neurospheres to study its effect. **B.** TGFβ1 and TGFβ2 protein levels secreted by endothelial cells are measured by ELISA. Data presented as mean ± SD

We wanted to address if the secretion of TGFβ was important for the GICs, so we pre-conditioned media for 3 days in contact with endothelial cells and then we cultured different patient-derived neurospheres. In all the cases, pre-conditioned media from endothelial cells activates the TGFβ pathway since we were able to observe phosphorylation of Smad2 (Figure 3.10 A) and activation of many TGFβ transcriptional targets such as *PAI1*, *SMAD7* or *LIF* (Figure 3.10 B). Pre-clearing the conditioned media with an anti-TGFβ blocking antibody or treating the neurospheres with the specific TβRI inhibitor LY2109761, prevented this phenothype. Interestingly, we observed a higher induction of *ID1* at both mRNA and protein levels when treating the cells with endothelial cell pre-conditioned media in comparison with TGFβ alone. This suggests that pre-conditioned media from endothelial cells contains some other growth factor that may cooperate with TGFβ in the induction of *ID1*.

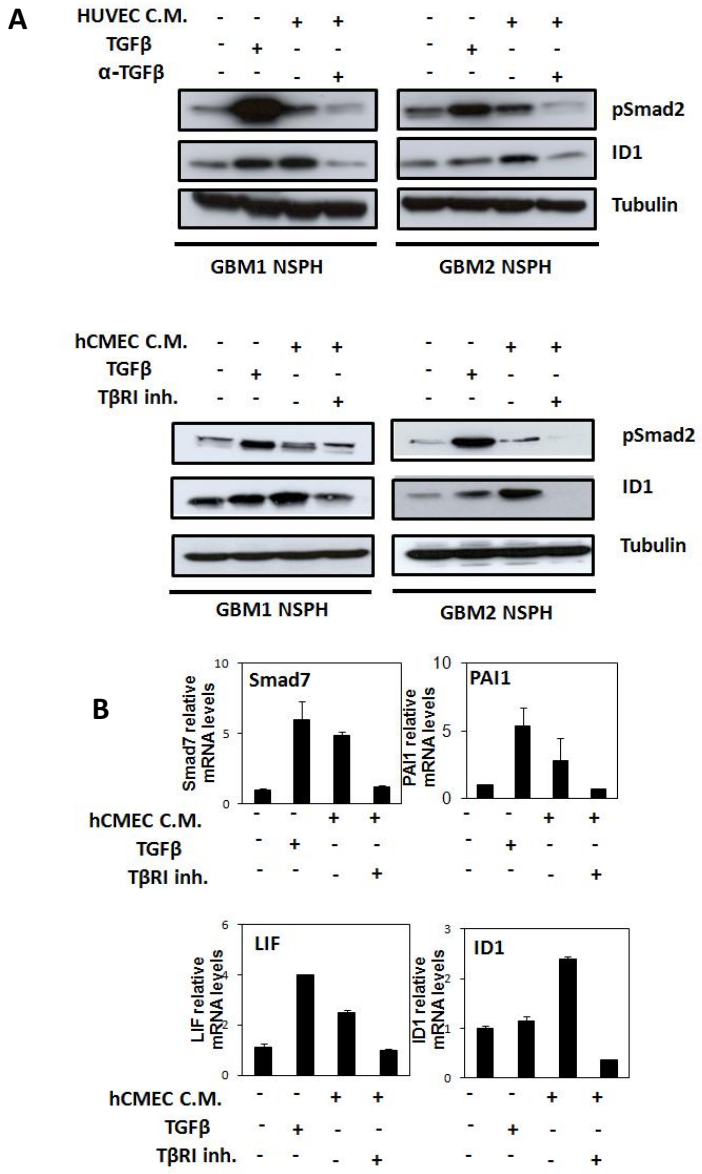


Figure 3.10. Pre-conditioned media from endothelial cells activates the TGFβ pathway in different patient-derived neurospheres. **A.** Neurospheres were incubated with TGFβ (100pM), pre-conditioned media or pre-conditioned media together with 2μM TβRI inhibitor. p-Smad2 and Id1 protein levels were assessed by immunoblotting. **B.** RNA was collected after treatment with TGFβ, pre-conditioned media or the combination of preconditioned media and TβRI inhibitor. Treatment with pre-conditioned media induces the expression of different TGFβ pathway targets in neurospheres (*LIF, SMAD7, PAI1* and *ID1*). Data are presented as mean ± SD.

TGFβ SECRETED BY ENDOTHELIAL CELLS IS NECESSARY TO MAINTAIN GICS AND THEIR PROPERTIES

We have previously demonstrated (Anido, Saez-Borderias et al. 2010) that $CD44^{high}$/Id1 positive population is enriched in GICs, and they are crucial for tumor initiation and recurrence. In order to elucidate the role of the TGFβ secreted by the endothelial cells in maintaining $CD44^{high}$/Id1 positive GICs, we treated several patient-derived neurospheres with pre-conditioned media from endothelial cells. In all the cases, conditioned media was able to increase the $CD44^{high}$ population of GICs and this effect was blocked either by an anti-TGFβ blocking antibody or by the TβRI inhibitor (Figure 3.11 A and B). Similarly, it also increased self-renewal capacity of GICs (Figure 3.12) which is readout of the tumor initiation capacity of the cells. Our results show that the TGFβ secreted by endothelial cells is not only capable to trigger the TGFβ pathway activity, but also it has an important role in maintaining the GIC population $CD44^{high}$/Id1 positive.

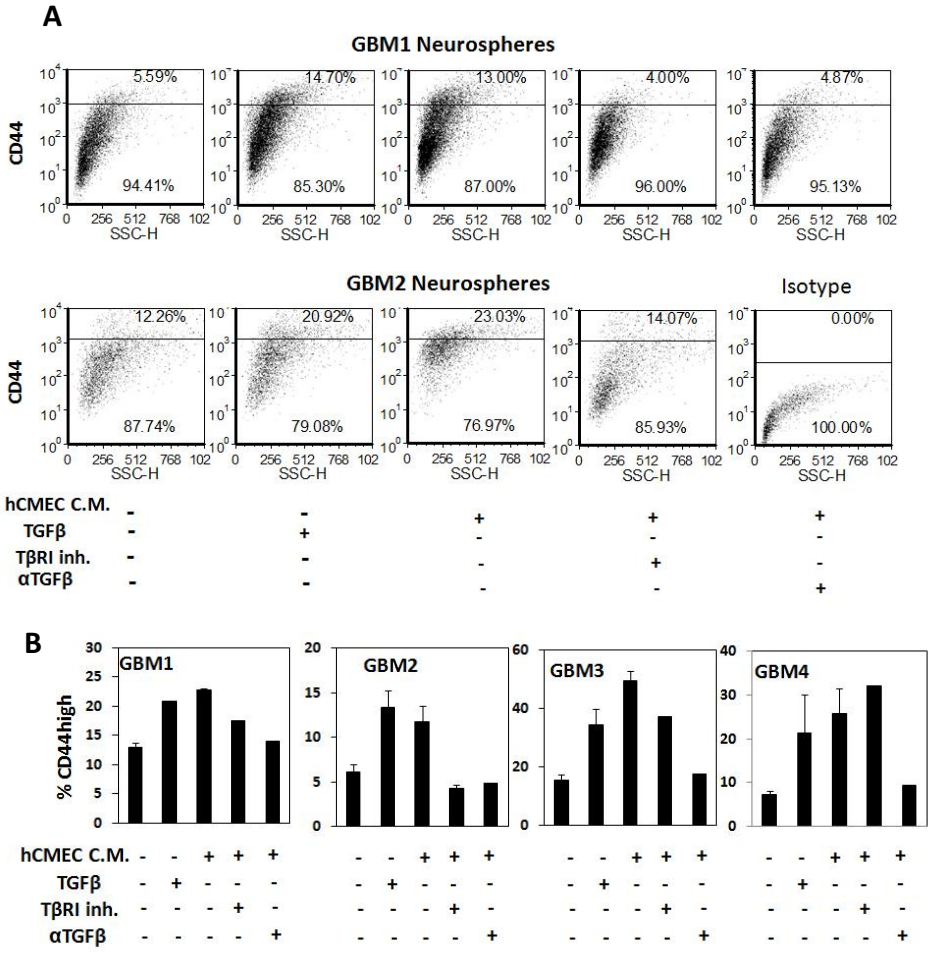


Figure 3.11. Endothelial cell pre-conditioned media increases the CD44^{high} GICs population in different patient-derived neurospheres. **A**. Neurospheres were incubated for 7 days with TGFβ (100pM), the pre-conditioned media or pre-conditioned media together with 2μM TβRI inhibitor. CD44 levels were assessed by FACS. **B.** Quantification of different experiments performed with different patient-derived neurospheres. Data presented as mean ± SD.

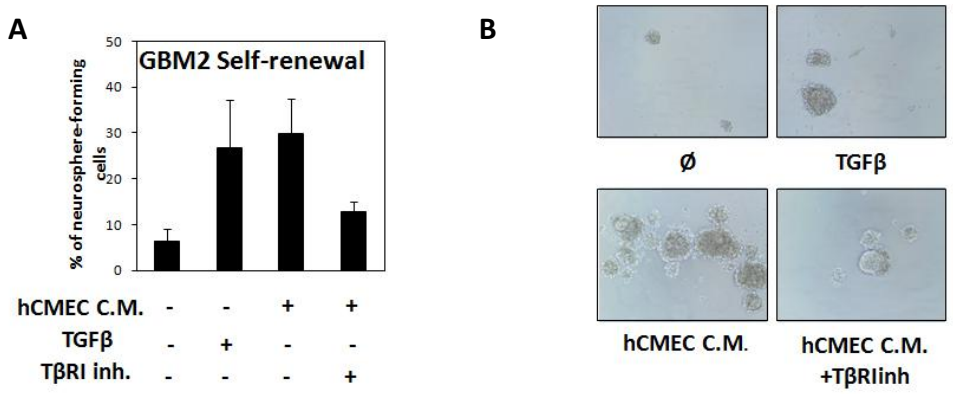


Figure 3.12. TGFβ pre-conditioned media from endothelial cells increases the self-renewal capacity of patient-derived neurospheres. **A.** GBM1 and GBM2 neurospheres were incubated for 7 days with TGFβ (100pM), endothelial cell pre-conditioned media or pre-conditioned media together with 2μM TβRI inhibitor. Neurospheres were disaggregated and counted and seeded at low density (4 cells/1μL). Newly formed neurospheres were counted after 10 days, in order to assess self-renewal capacity. Data presented as mean ± SD**. B.** Representative images are shown.

Furthermore, we wanted to assess the importance of TGFβ secreted from endothelial cells on the *in vivo* tumorigenic potential of GICs. We pretreated GBM-derived neurosphere culture with endothelial cell preconditioned media and we observed a significant increase in tumorigenic capacity (Figure 3.13 A and B). This effect was also blocked by pre-treating cells with TβRI inhibitor, demonstrating that the TGFβ secreted by endothelial cells was responsible for the increase of GIC's tumorigenic capacity.

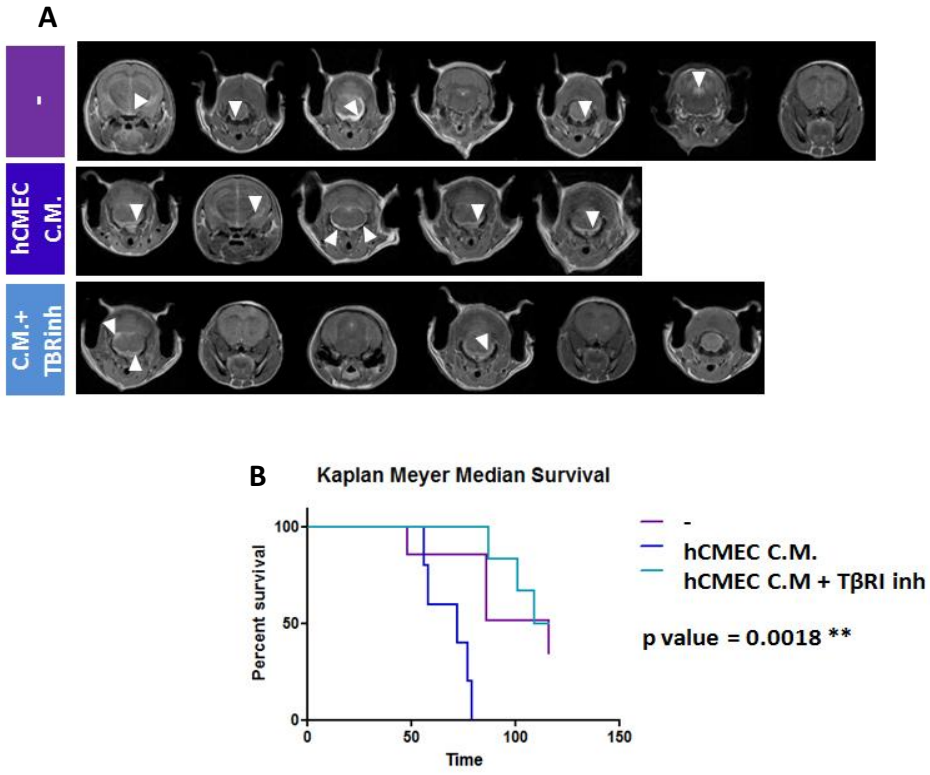
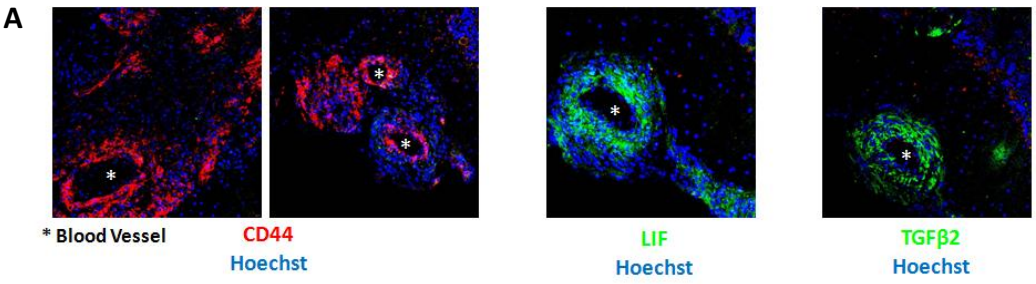


Figure 3.13. Endothelial cell pre-conditioned media increases tumorigenic capacity of patient-derived neurospheres. **A.** GBM1 neurospheres were incubated for 7 days with endothelial cell pre-conditioned media or pre-conditioned media together with 2µM TβRI inhibitor. Neurospheres were inoculated into immunocompromised mice (NOD/SCID) and tumor formation was assessed by MRI. **B.** Kaplan-Meier survival curves of control, hCMEC conditioned media (CM) or conditioned-media plus TβRI inhibitor groups.

IN VIVO TREATMENT WITH TβRI INHIBITOR DISRUPTS THE PERIVASCULAR NICHE FOR GICS

We were able to observe an enrichment of $CD44^{high}$ near the tumor vessels in our mouse xenograft model (Figure 3.14 A). We inoculated GBM-derived neurospheres and once mice developed tumors, we started treating them twice a day with an oral TβRI inhibitor (LY2109761). We observed that after 10 days of treatment, $CD44^{high}$ cells were no longer located near the blood vessels and tumors were remarkably smaller than the control ones with less CD44 overall staining (Figure 3.14 B and C).



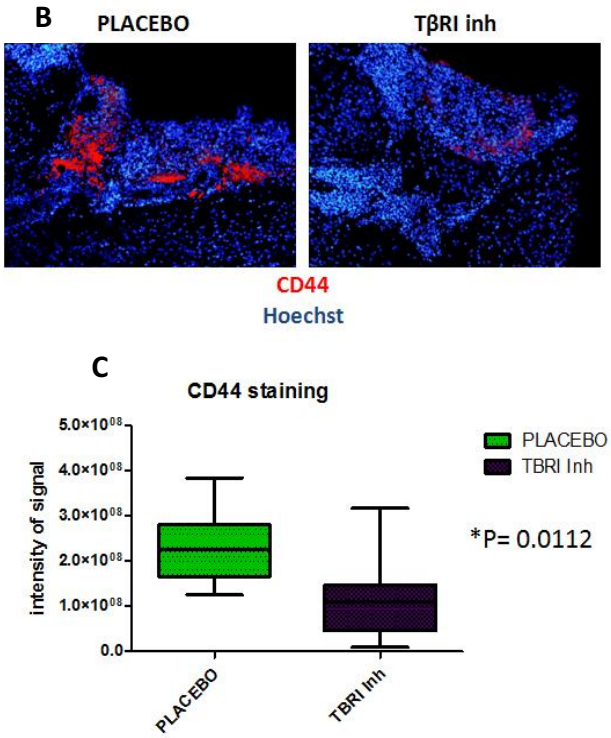


Figure 3.14. *In vivo* treatment with TβRI inhibitor disrupt the perivascular niche in glioma xenografts. **A**. (In the previous page) Immunofluorescence of patient-derived mouse orthotropic xenografts staining for CD44, LIF and TGFβ2. Nuclei were counterstained with Hoechst. **B.** Mice were orally treated with 75mg/kg of TβRI inhibitor for 10 days, twice a day. We observe a significant reduction in $CD44^{high}$ staining (right panel) compared to tumors in placebo-treated mice (left-panel). **C.** Quantification of CD44 intensity of staining. * = p value < 0.05.

Together these experiments demonstrate that endothelial cells secrete TGFβ and this is necessary to maintain GICs properties, such as $CD44^{high}$ and *ID1* expression, self-renewal capacity and tumorogenicity. It has been described (Gilbertson and Rich 2007) that GICs require different growth factors and cytokines to maintain their un-differentiated status and characteristics. Here we establish that TGFβ has an important role in the perivascular niche maintaining GICs characteristics (Figure 2.21 A).

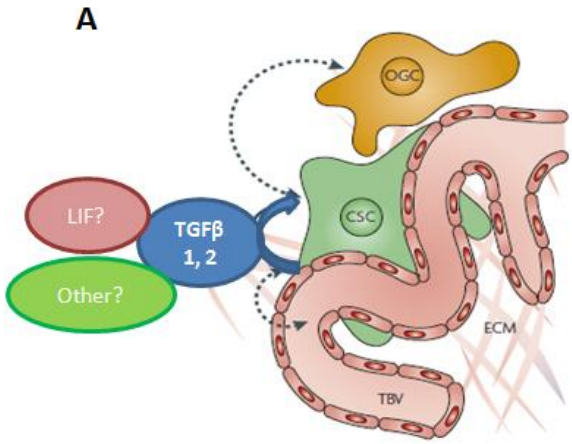


Figure 3.15. Schematic representation of the perivascular niche in glioma. Image adapted from (Gilbertson and Rich 2007). Tumor Initiating cells (or GICs) tend to be located in the proximity of tumor vessels because they receive different growth factors and cytokines. Among them, TGFβ 1 and 2 secreted by endothelial cells have an important role in maintaining GICs properties.

3. TGFβ MEDIATES RADIO-RESISTANCE OF GICS

IN VITRO IRRADIATION OF PATIENT-DERIVED NEUROSPHERES INCREASES CD44^{HIGH} GIC POPULATION

As described before, one of the main causes of therapeutic failure in glioma patients is recurrence shortly after treatment. It is suggested that this phenomenon occurs because conventional therapies target and efficiently kill the majority of the more differentiated cells within the tumor mass, but they do not target the Cancer Initiating Cell population. It has been demonstrated that GICs are resistant to DNA-damage induced by radiotherapy and chemotherapy (Bao, Wu et al. 2006; Rich 2007). Here we confirmed that the CD44^{high} GIC population that we have previously described and characterized is indeed resistant to gamma-irradiation. We irradiated *in vitro* neurospheres derived from 4 different patients (Figure 3.16 A) and in all cases we observed a significant increase in the CD44^{high} population after irradiation (Figure 3.16 B). This increase in CD44^{high} population was an early event as it was observed at 72 hours after the irradiation. This result demonstrates that GIC population CD44^{high} was resistant to irradiation *in vitro.*

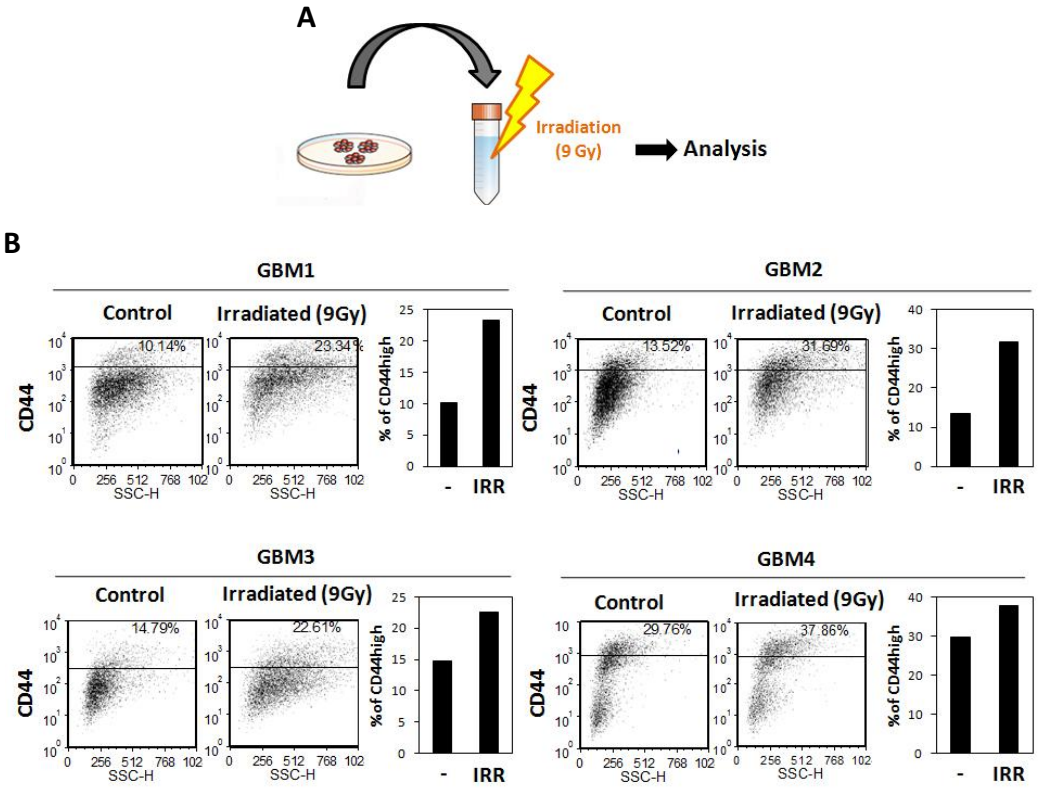
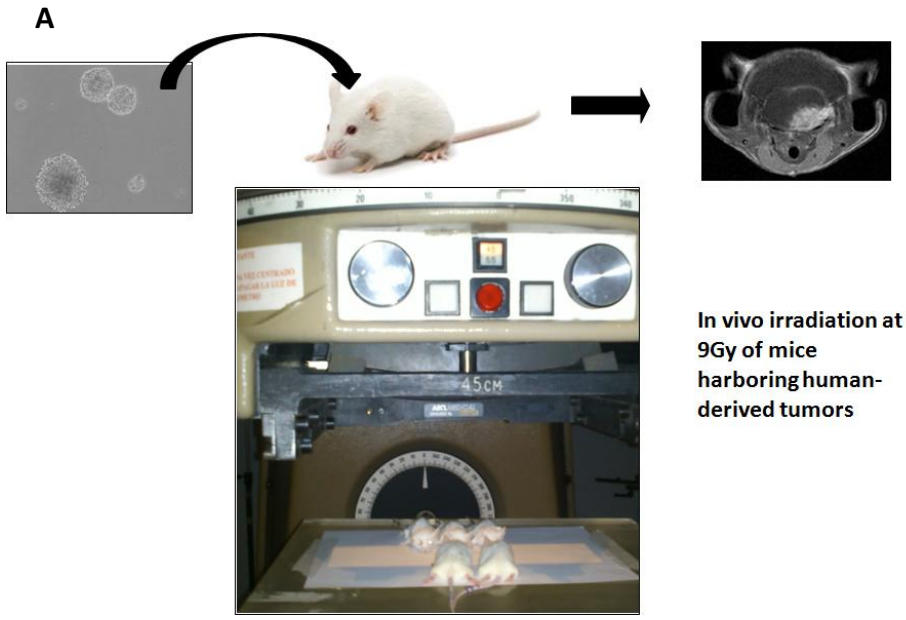


Figure 3.16. CD44^{high} GICs are radioresistant *in vitro.* **A.** Schematic representation of the experimental procedure. Patient-derived neurospheres cultures were irradiated at 9Gy *in vitro*. **B**. CD44^{high} levels were analyzed 3 and 5 days after irradiation.

We further validated our results *in vivo*. We irradiated mice harboring patient-derived tumors at a single dose of 9 Gy, which is equivalent to the dose given to glioma patients in radiotherapy treatment (Figure 3.17 A). We observed that irradiation induces severe apoptosis in tumor cells as assessed by TUNEL staining and Caspase 3 cleavage, an activation sign of an apoptotic pathway effectors protease (Figure 3.17 B left and middle panels). We also observed a significant enrichment in $CD44^{high}$/Id1 positive cells in irradiated tumors (Figure 3.17 B right). These results indicate that the $CD44^{high}$/Id1 positive GIC population is radioresistant both *in vitro* and *in vivo*.



B

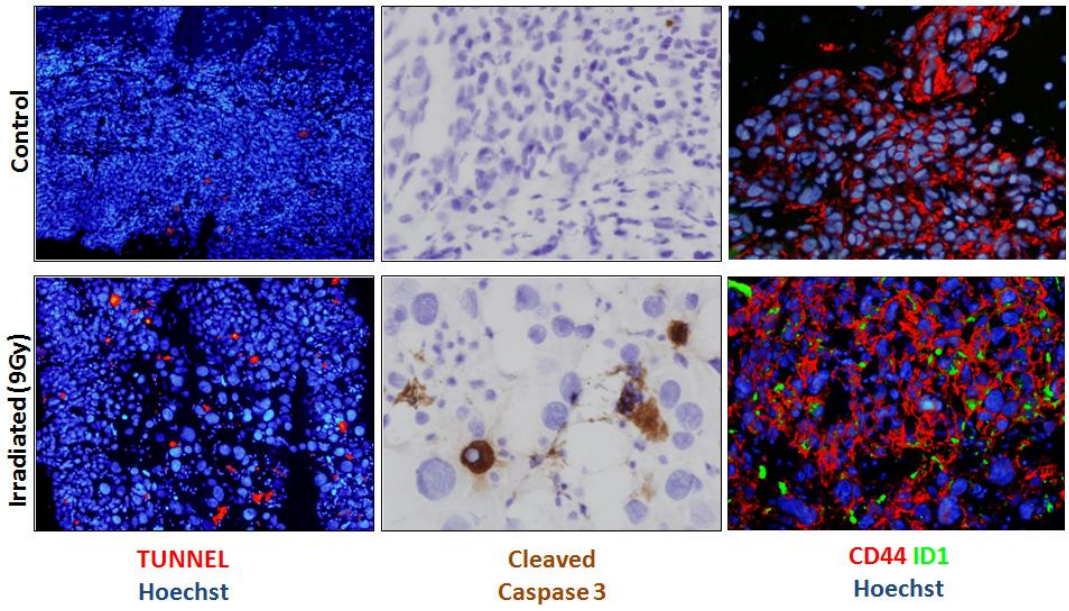


Figure 3.17. CD44^{high}/Id1+ GICs are radioresistant *in vivo.* **A**. (Previous page) Schematic representation of the experimental procedure. Mice harboring patient-derived tumors were irradiated at 9 Gy. **B.** Histological analysis of control and irradiated tumors was performed. TUNEL and Cleaved Caspase 3 (Left and middle panels) show irradiation-induced apoptosis. CD44 and Id1 levels were assessed by coimunofluorescence of frozen brains. Both CD44 and Id1 protein levels were increased after irradiation (right panel).

TREATMENT WITH TβRI INHIBITOR RADIOSENSITIZES GICS

It has been postulated that pathways important for GIC biology, may be good candidates for radiosensitizing GICs. Different signaling pathways have been demonstrated to confer radioresistance to CICs, for example Notch pathway has been shown that protects GICs from radiation-induced apoptosis (Wang, Wakeman et al. 2010). Previous work indicates that TGFβ may have a role in protecting CICs from radiation-induced DNA damage (Kim, Lebman et al. 2003; Dancea, Shareef et al. 2009; Zhang, Kleber et al. 2011; Hardee, Marciscano et al. 2012). We hypothesized that since TGFβ is important to maintain GICs and that the TβRI inhibitor decreases the $CD44^{high}$ population of GICs, it could be beneficial to combine both radiotherapy and inhibition of the TGFβ pathway to improve glioma treatment.

We pre-treated different patient-derived neurospheres with TβRI inhibitor LY2109761 for 7 days (Figure 3.18 A) and we confirmed that there was a significant reduction in $CD44^{high}$ levels. We then irradiated the same patient-derived neurospheres *in vitro* at a single dose of 9 Gy and we observed that, while control neurospheres increase the $CD44^{high}$ population after irradiation, TβRI inhibitor-treated neurospheres did not, maintaining the percentage of $CD44^{high}$ at less than 5% (Figure 3.18 B). We FACS-sorted $CD44^{high}$ and low populations and we were able to observe an increase in *ID1* mRNA levels after irradiation of patient-derived neurospheres that was restricted to the $CD44^{high}$ compartment. This increase in *ID1* expression is abolished by TβRI inhibitor treatment (Figure 3.18 C).

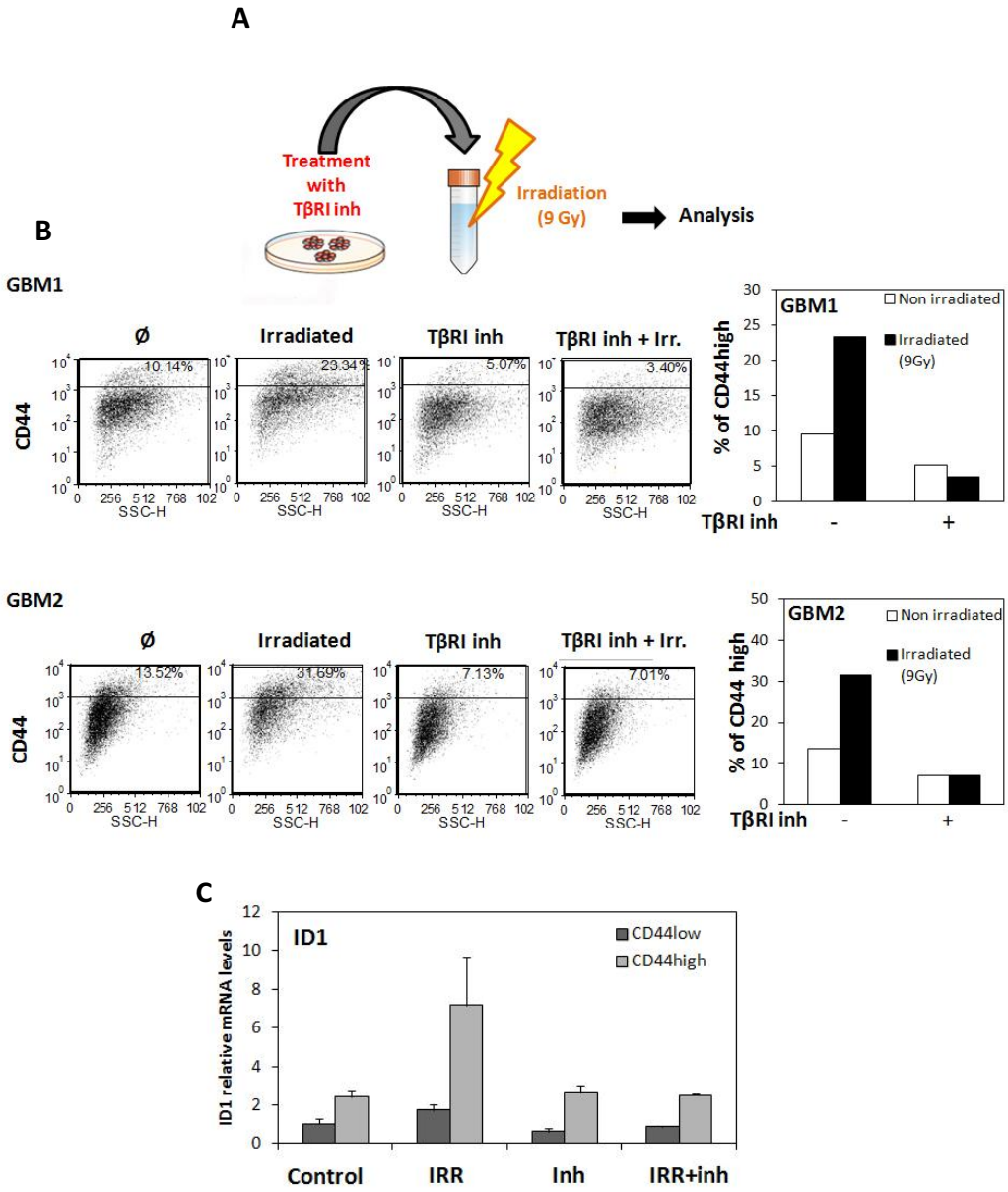


Figure 3.18. The increase in CD44^{high} population induced by irradiation was abolished by treatment with TβRI inhibitor. **A.** Schematic representation of the experimental procedure. Patient-derived neurospheres were pre-treated for 7 days with 2μM of TβRI inhibitor and then irradiated at 9 Gy. **B**. CD44 levels were compared after 5 days. **C**. CD44 high and low populations were FACS-sorted after irradiadiation and *ID1* mRNA levels were analyzed. *GAPDH* and *POLR2A* expression were used as a normalization control. Data are presented as mean ± SD.

We analyzed the cell viability of neurospheres after irradiation by monitoring the proliferation curves. Cells treated with radiotherapy alone were resistant, but in all the cases, combination of irradiation and TβRI inhibition efficiently blocked proliferation after 10 days of treatment (Figure 3.19).

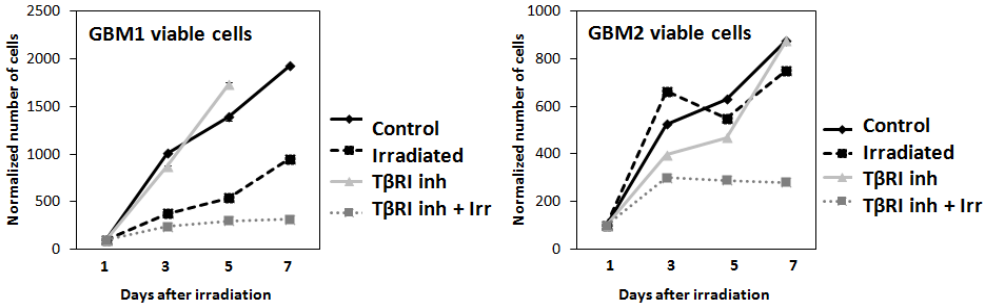


Figure 3.19. Combining irradiation and treatment with TβRI inhibitor efficiently decreases cell proliferation. Cells were irradiated at 9 Gy and treated with 2µM of TβRI inhibitor or left untreated. Cells were counted at different time points. Proliferation of alive cells was assessed using Propidium Iodide staining to distinguish dead cells.

We assessed apoptosis by monitoring the levels of cleaved PARP, a protein that is cleaved by effector caspases during the apoptotic response. We observed more apoptosis in the $CD44^{low}$ population than in $CD44^{high}$. After treatment with TBRI inhibitor, the $CD44^{high}$ population becomes more sensitive to irradiation-induced apoptosis, reaching the same apoptosis levels as the $CD44^{low}$ population. We confirmed that treatment with TBRI inhibitor is able to radiosensitize the $CD44^{high}$ population of GICs (Figure 3.20).

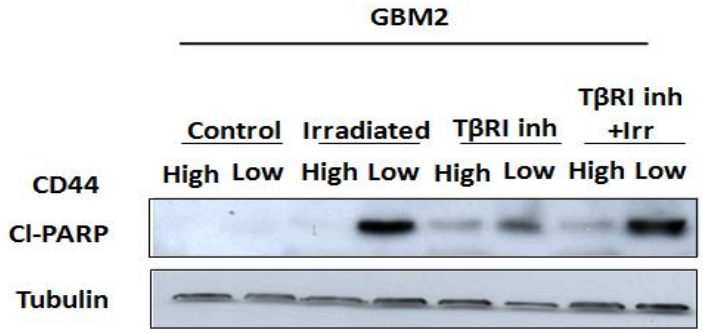


Figure 3.20. Combining irradiation and treatment with TβRI inhibitor increases apoptosis of $CD44^{high}$ GICs. GBM2-derived neurospheres were untreated or pre-treated for 7 days with 2μM of TβRI inhibitor and irradiated at a single dose of 9 Gy. They were FACS sorted depending on the CD44 levels and Cleaved-PARP levels were assessed by immunoblott as a readout of apoptosis. Tubulin levels were used as a loading control.

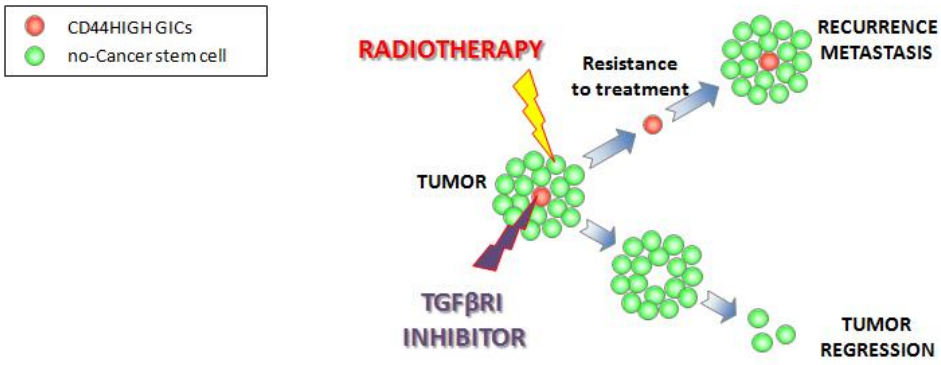


Figure 3.21. Summary of our hypothesis. Conventional therapies such as radiotherapy, target the non-cancer initiating cell population, leading to further resistance and relapse. Targeting CICs (GICs) with TβRI inhibitor in combination with radiotherapy efficiently decreases cell proliferation and may prevent tumor relapse.

4. RUNX1 IS A MEDIATOR OF THE TGFβ ONCOGENIC EFFECT IN GLIOMA

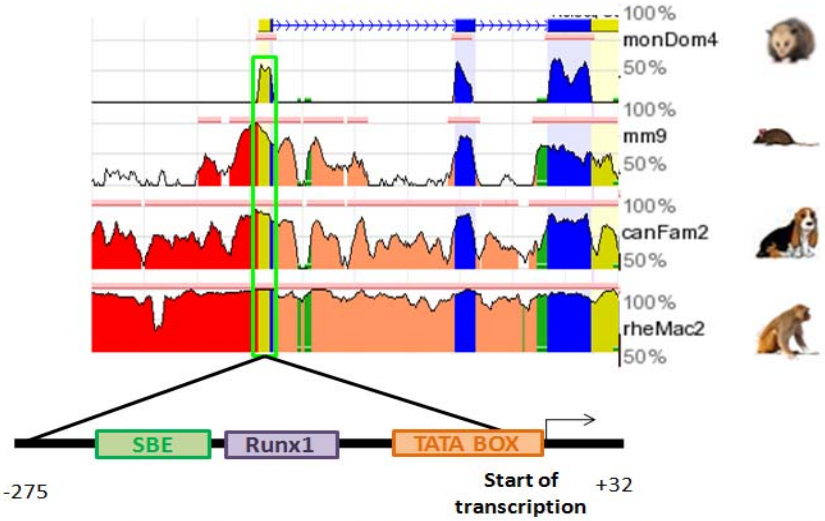
Our group has previously described some critical aspects of the TGFβ oncogenic effect in glioma. First of all, TGFβ is promoting glioma cell proliferation through the induction of the growth factor PDGFB (Bruna, Darken et al. 2007). Furthermore, it increases Glioma Initiating Cell self-renewal through the induction of the cytokine LIF (Penuelas, Anido et al. 2009) and also through the induction of the Sry-related HMG-box factors Sox2 and Sox4 (Ikushima, Todo et al. 2009). We were interested in underlying the molecular mechanisms of this TGFβ oncogenic effect in glioma, with especial interest in finding new mediators that may explain this dual role of TGFβ in cancer. Most of our work is focused in the study of how TGFβ regulates GICs, especially for its therapeutic implications discussed in the introduction and further commented on the discussion. One of the main mediators of the TGFβ oncogenic effect is LIF, and we wanted to further elucidate the mechanism of induction of LIF cytokine by TGFβ in glioma.

IN SILICO ANALYSIS OF THE TGFβ-RESPONSIVE REGION OF THE LIF PROMOTER REVEALED TWO PUTATIVE RUNX1 BINDING SITES

We have previously characterized the *LIF* promoter region and the Smad Binding Element (SBE) (Penuelas, Anido et al. 2009). We have cloned the wild-type promoter region of *LIF* (600bp) into a luciferase reporter vector and then identified the TGFβ-responsive region by subcloning different

fragments as detailed in Material and Methods. We demonstrated that the -300 bp region of LIF 5'-UTR was necessary for the TGFβ response and identified a SBE (-115 bp) in the region. Mutation of the SBE motif abolished the induction of LIF by TGFβ.

It is well known that Smads have low affinity for DNA unless they cooperate with other transcription factors (Massague, Seoane et al. 2005). We were interested in finding which Transcription Factor (TF) may cooperate with Smads in TGFβ-mediated induction of LIF. We analyzed the promoter region of *LIF* and search for TF binding sites. First of all, we compared the promoter region of different species (*Rhesus macacus*, *Cannis familiaris*, *Mus musculus* and *Monodelphis domestica*) and aligned the promoter sequences using ClustalW. We found that there were many conserved regions throughout the evolution of the LIF promoter region, suggesting that there was a negative selection pressure avoiding any mutations. This is typical for TF binding sites, as they are important for gene expression and they are usually conserved during evolution. We then used MEME and TRANSFAC to search for other TF binding sites in the *LIF* promoter (see Materials and Methods). Interestingly, we found two binding sites for Runx1 TF; one of them near the SBE. It is known that Smad TF and Runx1 TF can bind together and cooperate in the induction of many transcription responses (Hanai, Chen et al. 1999; Zaidi, Sullivan et al. 2002; Ito and Miyazono 2003). We then postulated that Runx1 might be cooperating with Smads in the LIF induction by TGFβ.



CGGGACAAGCCAGGCAGGAAAACCACAGGGCGTTTTGTCGAAGGCTTCATTATAATTTTATCAATCAAATTCTTAGAAGAGGGAAAAAGTCTG
TTCTCCCCACCCTCCCCCCTCACTCGTCCCCCCCCTTCACTCTCACTTTCTTCCATTCATAATTTCCTATGATGCACCTCAAACAACTTCCTGGA
CTGGGGATCCCGGCTAAATATAGCTGTTTCTGTCTTACAACACAGGCTCCAGTATATAAATCAGGCAAATTCCCCATTTGAGCATGAACCTCT
GAAAACTGCCGGCATCTGAGGTTTCCT

Figure 3.22. *LIF* promoter region was compared between different species. Schematic representation of *LIF* promoter region showing Smad Binding Element (SBE) and Runx1 Binding Site. Nucleotide sequence is shown with SBE highlighted in green and Runx1 Binding Site in purple. TATA box is shown in orange.

RUNX1 BINDING SITE MUTATION ABOLISHES TGFβ- MEDIATED INDUCTION OF LIF

To study the importance of Runx1 TF as a cofactor of Smads in the induction of LIF by TGFβ, we used site directed mutagenesis to introduce two point mutations in the Runx1 binding site closer to SBE in the LIF promoter. We performed a luciferase reporter assay and we were able to observe that, while the wild-type *LIF* promoter was activated after TGFβ treatment, the mutation of either SBE or Runx1 Binding Site abolished the TGFβ activation of *LIF* promoter (Figure 3.23 B).

We also transfected 293T cells, which do not show LIF activation upon TGFβ treatment and do not express significant levels of *RUNX1*, with the *LIF* promoter reporter vector. We do not observe any activation of the reporter with TGFβ treatment, but when we simultaneously co-transfected a *RUNX1* expression vector (pCMV-flag Runx1) we observed a significant increase in *LIF* promoter activation in basal conditions and in response to TGFβ treatment (Figure 3.23 C).

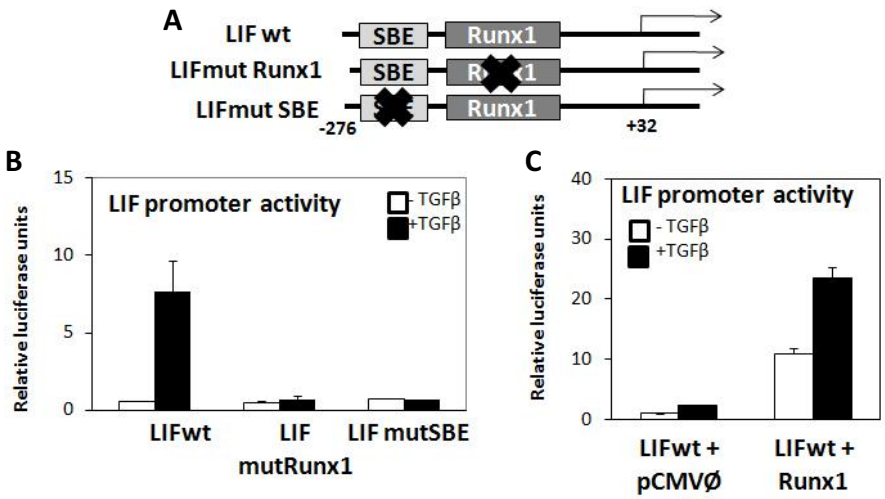


Figure 3.23. *LIF* promoter reporter assay (from previous page) **A**. *LIF* promoter was mutated at Runx1 binding site and Smad Binding Element, as shown in the scheme. **B**. Luciferase reporter assay was performed in A172 glioma cells. *LIF* wild-type promoter luciferase reporter vector and two mutants (Runx1 binding site mutant and SBE mutant) were transfected together with Renilla-TK expressing vector. Cells were treated for 24 hours with 100pM of TGFβ or left untreated. Cells were lysed and luciferase activity was measured as a read out of promoter activation. **C**. A reporter construct expressing either wild-type or mutated *LIF* promoter was transfected in 293T cells, together with or without *RUNX1* overexpression vector. Luciferase was measured as readout of *LIF* promoter activity was assessed after 24 hours of TGFβ treatment. Renilla-TK was used as a transfection control and luciferase activity was normalized. Data are presented as mean ± SD.

RUNX1 TRANSCRIPTION FACTOR PHYSICALLY BINDS TO THE LIF PROMOTER REGION

To analyze if Runx1 is physically associated to the putative binding site described above, we performed Chromatin ImmunoPrecipitation (ChIP) in U373 glioma cells. We immunoprecipitated Runx1 with a specific antibody and then performed quantitative Real Time PCR of the chromatin crosslinked to the transcription factor. We found binding of Runx1 to LIF promoter region and an enhancement of the binding upon TGFβ treatment (Figure 3.24).

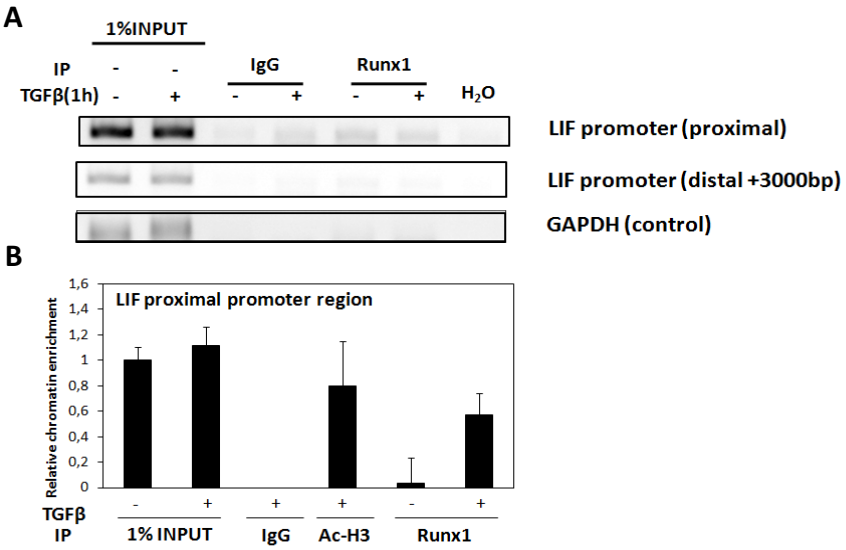


Figure 3.24. Runx1 ChIP was performed in U373 cells. Cells were treated with 100pM TGFβ or untreated for 1 hour. Chromatin-protein crosslinked complexes were immunoprecipitated using the corresponding antibodies. Normal IgG was used as a negative control and Ac-Histone 3 was used as a positive control. **A.** PCR of immunoprecipitated chromatin was performed using primers designed for the promoter region of *LIF* and a distal (+3000 bp) region of *LIF* promoter. *GAPDH* promoter was used as a negative control. **B.** Enrichment of chromatin bound to Runx1 TF was measured by qPCR and normalized by 1% chromatin input. Data is presented as mean ± SD.

RUNX1 TRANSCRIPTION FACTOR IS NECESSARY FOR LIF INDUCTION BY TGFβ

To further confirm the importance of Runx1 TF in TGFβ-mediated induction of *LIF*, we performed a knock-down by silencing RNA (siRNA) in glioma cells (U373). When Runx1 was decreased, the induction of *LIF* by TGFβ was also decreased (Figure 3.25 A and B). We also performed a stable knock-down with a short hairpin RNA (shRNA) in U373 glioma cells. Again, cells with decreased expression of *RUNX1* had a reduced induction of *LIF* by TGFβ (Figure 3.25 C and D), showing that indeed Runx1 was necessary for *LIF* induction by TGFβ (at mRNA and protein levels) (Figure 3.25 C, D, E and F).

We then decided to study this mechanism in a model that more closely resembles the human disease, so we decided to study the role of Runx1 in patient-derived neurospheres. To do so, we did a screening for Runx1 levels in different of our patient-derived cultures as well as in patient-derived xenografts. We selected the neurospheres with higher expression of *RUNX1* and *LIF* both in culture and *in vivo*. We used lentiviral miRNA-adapted shRNA to knock-down *RUNX1* in two different patient-derived neurosphere cultures. In both cases, the knock-down of *RUNX1* transcription factor significantly decreased the induction of LIF by TGFβ (Figure 3.26).

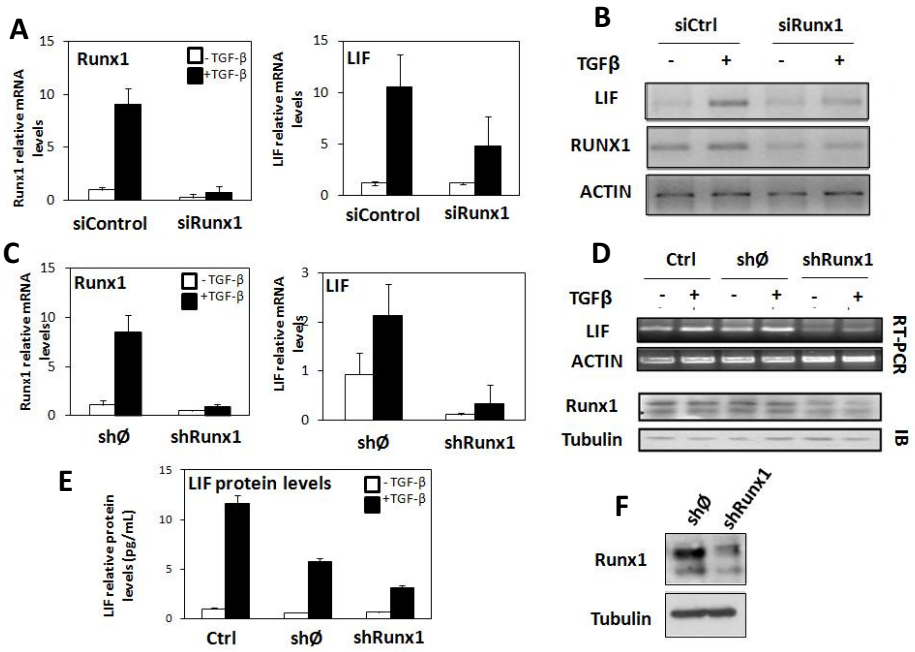


Figure 3.25. Runx1 Transcription Factor is necessary for *LIF* induction by TGFβ in glioma cell line. **A.** U373 cells were transfected with *RUNX1* siRNA to knock-down *RUNX1*. *LIF* and *RUNX1* expression were measured by qRT-PCR and normal PCR (**B**) after treatment with or without 100pM of TGFβ for 3 hours. **C**. U373 cells were stably infected with lentivirus with short-hairpin RNA targeting *RUNX1* mRNA to perform a stable *RUNX1* knock-down. *LIF* and *RUNX1* mRNA levels were measured by qRT-PCR with or without treatment with 100pM of TGFβ for 3 hours. **D.** Regular RT-PCR and immunoblot showing a decrease in *LIF* expression and in Runx1 protein levels**. E.** LIF protein levels were measured by ELISA. **F.** Immunoblot showing Runx1 decrease caused by the stable short hairpin RNA. Data are presented as mean ± SD.

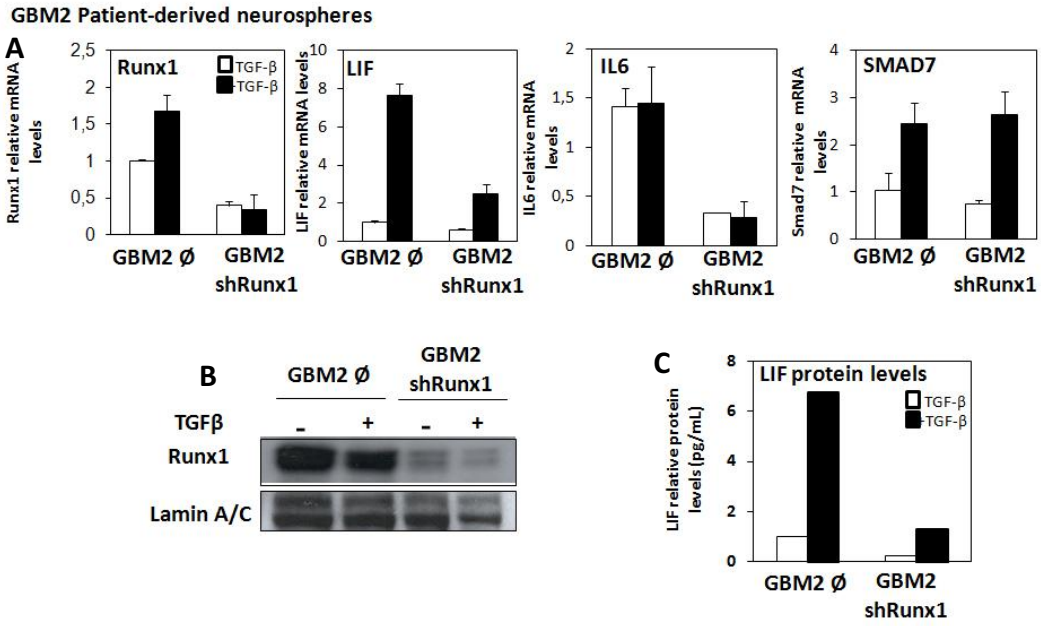


Figure 3.26. Runx1 Transcription Factor is necessary for LIF induction by TGFβ in patient-derived neurospheres. **A.** GBM-derived neurospheres were stably infected with a short hairpin RNA targeting *RUNX1*. *RUNX1, LIF, IL6* and *SMAD7* mRNA levels were measured by qRT-PCR after 3 hours of treatment with or without TGFβ (100pM). **B.** Immunoblot showing the decrease in Runx1 protein levels with the short-hairpin. **C.** LIF protein levels were measured by ELISA. Data are presented as mean ± SD.

OVEREXPRESSION OF RUNX1 IS SUFFICIENT TO INDUCE LIF EXPRESSION

We used the opposite approach to demonstrate the role of Runx1 as a mediator of *LIF* induction. We overexpressed *RUNX1* in two different glioma cell lines (U373 and U87) and in both cases there was a significant increase in basal and TGFβ-induced *LIF* expression and protein secretion (Figure 3.27 A and B).

We also overexpressed *RUNX1* in patient-derived neurospheres (GBM4) that normally express lower levels *RUNX1*. As observed in immortalized cell lines, GBM-derived neurospheres overexpressing *RUNX1* showed an increase in *LIF* expression and induction by TGFβ as well as LIF secretion measured by ELISA (Figure 3.27 C and D).

RUNX1 AND LIF LEVELS CORRELATE IN GBM PATIENTS

We analized 347 Samples from TCGA using GeneSapiens and we observed a significant correlation between *RUNX1* and *LIF* mRNA levels in GBM patients with a rho value of 0.404 and a statistically significant p value ($p<0.001$) (Figure 3.28 A). We then wanted to see if Runx1 was expressed in GBM samples. We performed IHC and co-imunefluorescence of LIF and Runx1. As antibodies were from the same species we used consecutive slices of the same tumor. We were able to observe a colocalization in the cells expressing Runx1 and LIF in some of the GBM patients analized (Figure 3.28B). Interestingly, cells that express LIF and Runx1 were also $CD44^{high}$ and located in the periphery of tumor vessels. Thus, Runx1 and LIF might be expressed by GICs located in the perivascular niche in glioblastoma patients.

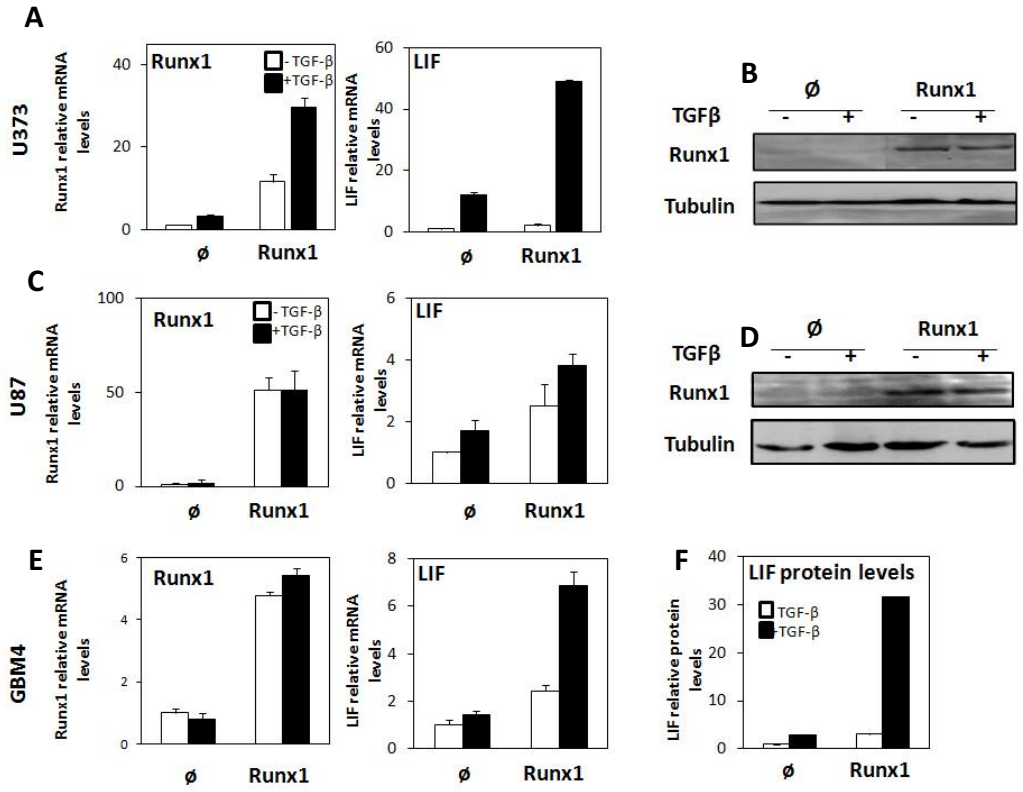


Figure 3.27. Overexpression of *RUNX1* is sufficient to induce *LIF* expression**. A.** U373 glioma cells were stably infected with a lentivirus overexpressing *RUNX1* TF. *RUNX1* and *LIF* mRNA levels were measured by qRT-PCR after 3 hours of treatment with or without TGFβ (100pM). **B.** Immunoblot showing Runx1 protein levels. **C.** U87 glioma cells were stably infected with a lentivirus overexpressing *RUNX1* TF. *RUNX1* and *LIF* mRNA levels were measured by qRT-PCR after 3 hours of treatment with or without TGFβ (100pM). **D.** Immunoblot showing Runx1 protein levels. **E.** GBM-derived neurospheres were stably infected with a lentivirus overexpressing *RUNX1*. **F.** Levels of LIF protein secreted to the media were measured by ELISA. Data are presented as mean ± SD.

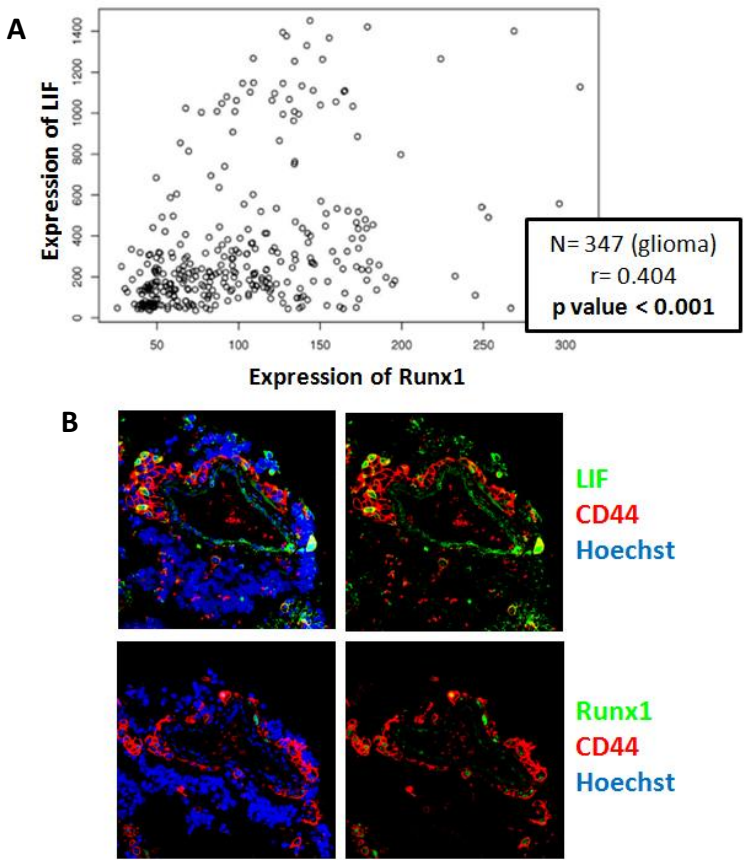
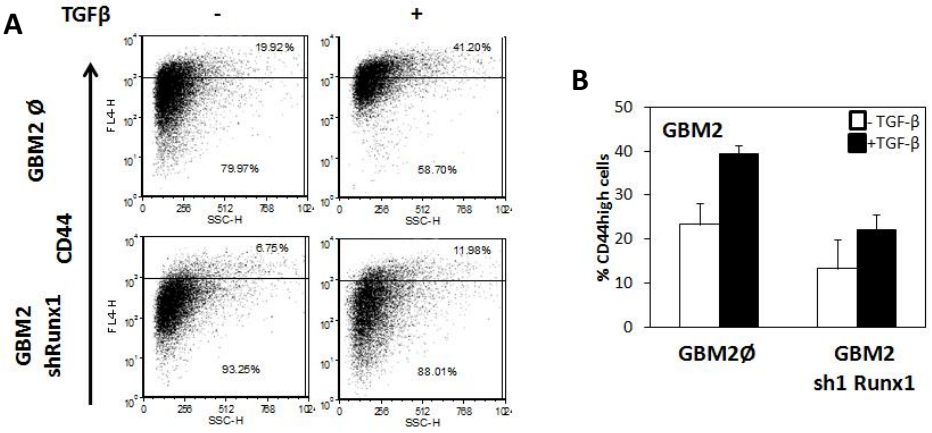


Figure 3.28. Runx1 and LIF levels correlate in GBM patients. **A.** 347 glioma samples were analyzed for gene expression. Correlation between *LIF* and *RUNX1* mRNA expression is shown (rho = 0.4, p value < 0.001). Data obtained from GeneSapiens database. **B.** Different glioma sections were stained with LIF, CD44 and Runx1 antibodies, as shown. Nuclei were counterstained with Hoechst. Two consecutive sections are shown to see the correlation of expression within the same tumor area. Representative images from a GBM patient are shown.

RUNX1 IS NECESSARY TO MAINTAIN CD44^{HIGH}/ID1 POSITIVE POPULATION AND SELF-RENEWAL CAPACITY OF GICS

We knocked-down *RUNX1* in two different patient-derived neurospheres and we observed a significant decrease in the CD44^{high} population and in the induction by TGFβ (Figure 3.29 A and B).

We did the opposite approach and we overexpressed *RUNX1* full-length isoform in a glioma-derived neurosphere culture, which normally express lower levels of Runx1. When we overexpressed *RUNX1*, the CD44^{high} levels significantly increase as well as the induction by TGFβ (Figure 3.30).



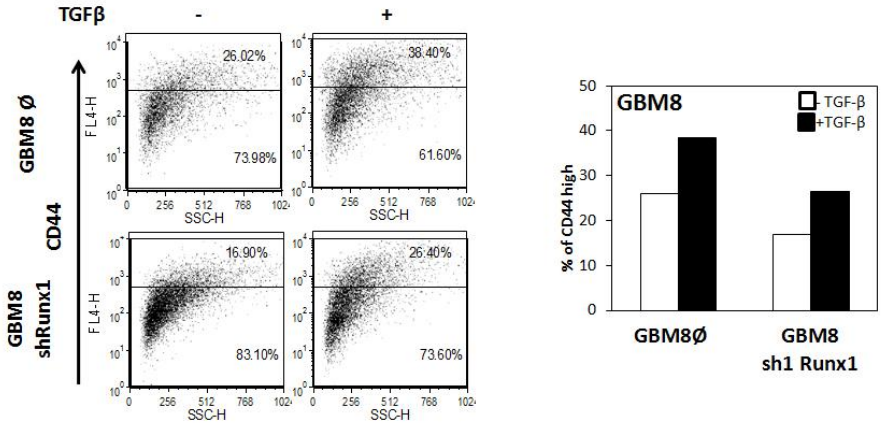


Figure 3.29. Runx1 is necessary to maintain CD44^{high}/Id1 positive GICs**. A**. (In the previous page) GBM2 patient-derived neurospheres were infected with short hairpin targeting *RUNX1* expression. Cells were treated with 100pM of TGFβ for 5 days or left untreated. Percentage of CD44^{high} cells was measured by FACS cytometry. **B**. Mean of different experiments. Data are presented as mean ± SD. **C**. GBM8 patient-derived neurospheres were infected with short hairpin targeting *RUNX1* expression. Cells were treated with TGFβ for 5 days or left untreated. Percentage of CD44^{high} cells was measured by FACS cytometry. **D**. Mean of different experiments.

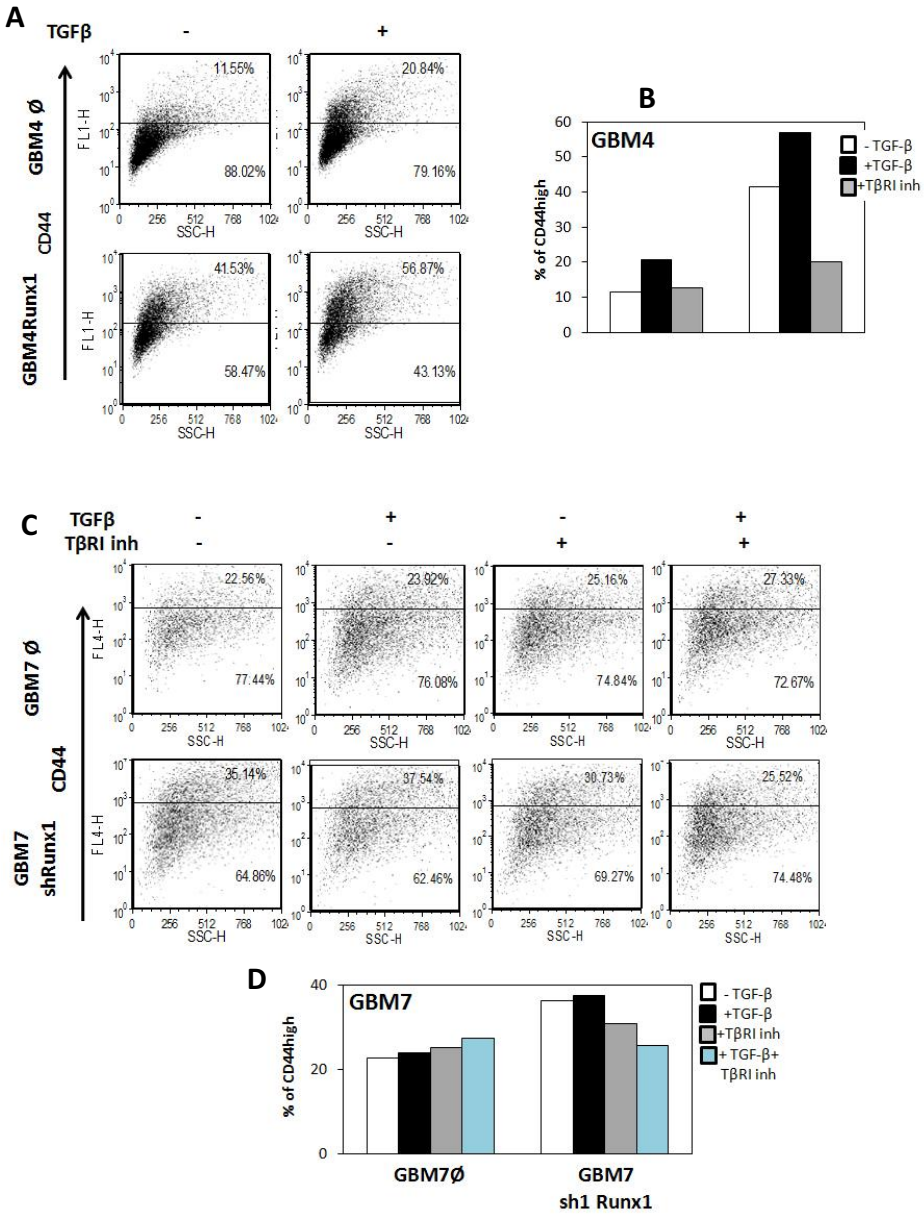


Figure 3.30. *RUNX1* overexpression increases the proportion of GICs in neurosphere cultures. **A**. GBM4 patient-derived neurospheres were infected with a lentivirus overexpressing *RUNX1*. Cells were treated with TGFβ for 5 days or left untreated. Percentage of $CD44^{high}$ cells was measured by FACS cytometry. **B**. Mean of different experiments. **C**. GBM7 patient-derived neurospheres were infected with a lentivirus overexpressing *RUNX1*. Cells were treated with 100pM TGFβ and TβRI inhibitor for 5 days or left untreated. Percentage of $CD44^{high}$ cells was measured by FACS cytometry. **D**. Mean of 3 independent experiments.

We hypothesized that, since *LIF* induction by TGFβ was necessary to increase GIC self-renewal, Runx1 may be important for this process. Therefore, we analyzed the self-renewal capacity of neurospheres with the knock-down of *RUNX1*. Cells with a *RUNX1* knock-down exhibited reduced self-renewal capacity demonstrating the importance of Runx1 to maintain GIC properties (Figure 3.31).

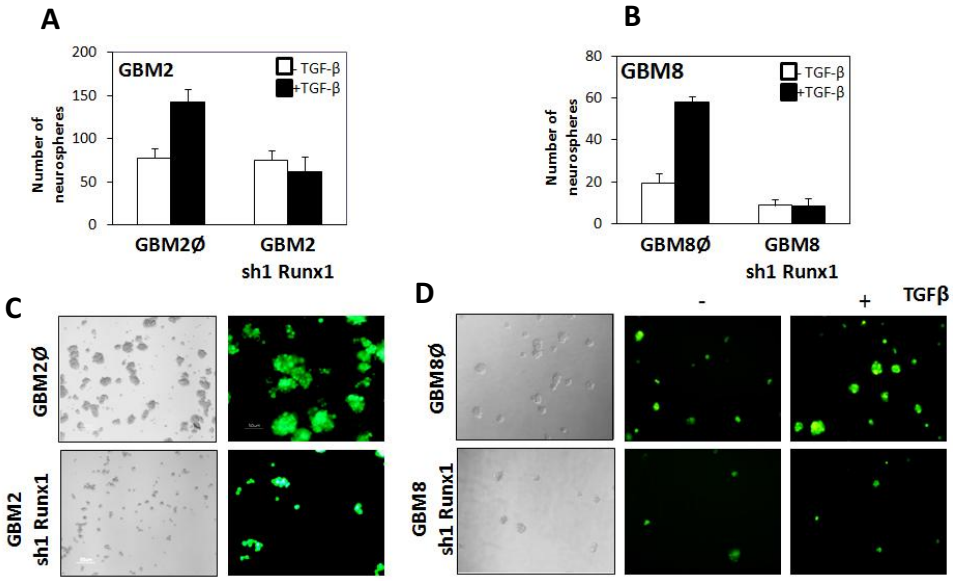


Figure 3.31. Runx1 is necessary for GIC self-renewal**. A.** GBM2 patient-derived neurospheres were infected with a lentivirus expressing a short hairpin RNA targeting *RUNX1*. Cells were treated for 5 days with 100pM TGFβ or left untreated. Neurospheres were dissociated, counted and equal numbers of cells were plated. Newly generated neurospheres were counted after 10 days. **B.** GBM8 patient-derived neurospheres were infected with a lentivirus expressing a short hairpin RNA targeting *RUNX1*. Cells were treated for 5 days with TGFβ (100pM) or left untreated. Neurospheres were dissociated, counted and equal numbers of cells were plated. Newly generated neurospheres were counted after 10 days. **C, D.** Representative images are shown for each condition. Phase contrast in the left panels and green fluorescence in the right panels. Data are presented as mean ± SD

We overexpressed *RUNX1* in patient-derived neurospheres and assessed their self-renewal capacity with or without *RUNX1* overexpression. We observed that *RUNX1* overexpression significantly increases self-renewal of patient-derived neurospheres, further supporting the role of Runx1 as a mediator of *LIF* induction by TGFβ (Figure 3.32 E and F).

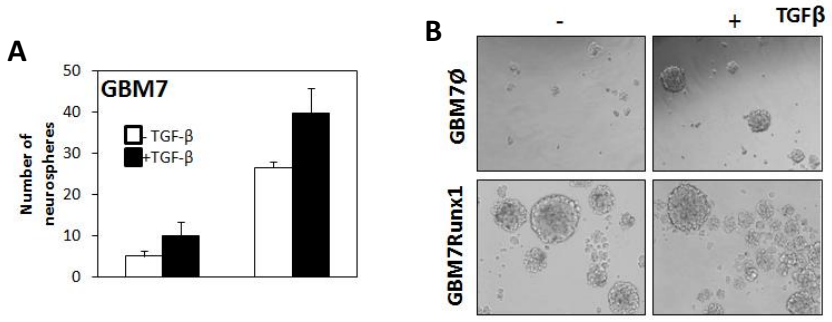


Figure 3.32 Overexpression of *RUNX1* increases GIC self-renewal. **A.** GBM7 patient-derived neurospheres were infected with a lentivirus overexpressing Runx1. Cells were treated for 5 days with 100pM TGFβ or left untreated. Neurospheres were dissociated and counted and equal numbers of cells were plated. Newly generated neurospheres were counted after 10 days. **B**. Representative images of newly formed neurospheres. Data are presented as mean ± SD.

RUNX1 IS NECESSARY TO MAINTAIN GICS IN AN UNDIFFERENTIATED STATE

We have previously reported that LIF was necessary to maintain GICs in an undifferentiated state, expressing stem cell markers such as *NESTIN*, *MUSASHI-1* or *SOX2* and inhibiting the differentiation towards neuronal, astrocytic or oligodendrocytic lineages (Penuelas, Anido et al. 2009).

We knocked-down *RUNX1* in patient-derived neurospheres and we assessed the levels of stem markers (*NESTIN* and *SOX2*) and differentiation markers (*GFAP* for Astrocytic lineage).

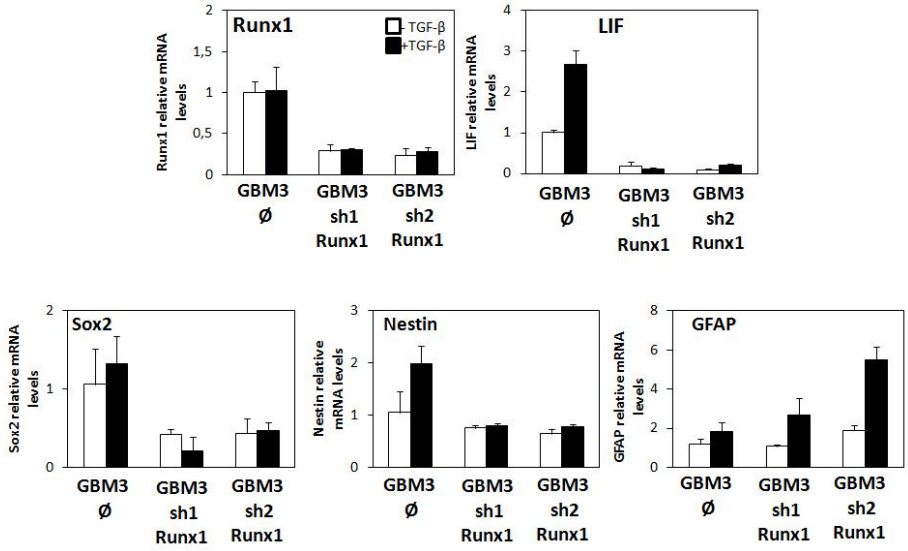


Figure 3.33. Runx1 is necessary to maintain GICs in an undifferentiated state. GBM3 patient-derived neurospheres were infected with a lentivirus expressing a short hairpin targeting *RUNX1*. Cells were treated with 100pM TGFβ for 5 days or left untreated. mRNA levels of different stemness or differentiation markers were measured by qRT-PCR. Data are presented as mean ± SD.

We observed that neurospheres with *RUNX1* knock-down showed decreased levels of stem markers and a significant increase in GFAP expression, suggesting that Runx1 may be preventing the differentiation of GICs towards an astrocytic phenotype (Figure 3.33). Interestingly, other differentiation markers such as oligodendrocytic marker O4 or neuronal marker Tuj1 were not increased.

We further confirmed our results by immunofluorescence staining (Figure 3.34). Knock-down of *RUNX1* decreased the expression of Nestin stem marker while increases the expression of GFAP astrocytic differentiation marker.

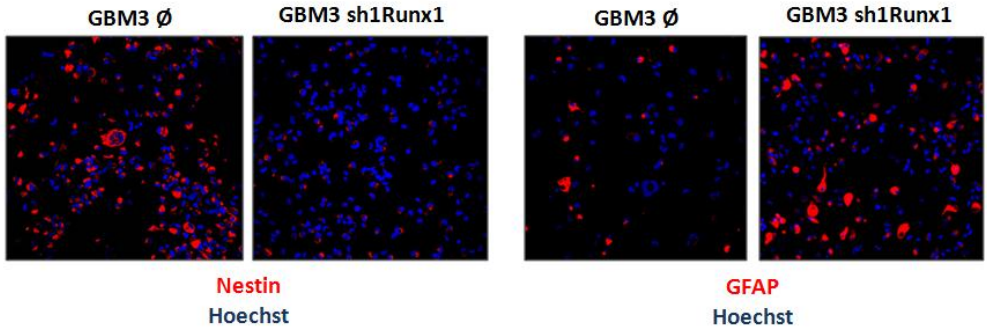


Figure 3.34. Runx1 is necessary to maintain GICs in an undifferentiated state. GBM3 patient-derived neurospheres were infected with a lentivirus expressing a short hairpin targeting *RUNX1*. Cells were treated with TGFβ for 5 days or left untreated. Immunofluorescence was performed for Nestin and GFAP. Nuclei were counterstained with Hoechst.

RUNX1 IS NECESSARY FOR THE MESENCHYMAL PHENOTYPE OF GBM

It has been described that GBM can be divided in four different subclasses: Classical, Neural, Proneural and Mesenchymal, the latter class being the one with the worst prognosis (Phillips, Kharbanda et al. 2006). A bioinformatic approach was used to predict the transcription factors that are master regulators of the mesenchymal sub-type of GBM. Interestingly, Runx1 was among the 6 TF signature that they postulate is driving the mesenchymal transformation of GBM (Carro, Lim et al. 2010). Furthermore, analyzing the gene-expression data of the Mesenchymal phenotype GBMs, *RUNX1* and *LIF* were significantly up-regulated by 2.68 fold and 8.49 fold change respectively in Mesenchymal GBMs compared to other subtypes of GBM.

To further confirm the role TGFβ in the mesenchymal transformation, we treated U373 glioma cells for 7 days with TGFβ to promote trans-differentiation. We analyzed different genes from the mesenchymal signature such as *RUNX1, LIF, ANGPTL-4, YKL-40, PAI1 (SERPINE1)* and some proneural markers (*BCAN* and *OLIG-2*) (Phillips, Kharbanda et al. 2006). We observed that TGFβ increased the expression of the mesenchymal markers *RUNX1*, *LIF* and *ANGPTL-4* and that treatment with TβRI inhibitor decreased their expression (Figure 3.35). No changes were observed in *YKL-40* expression after treatment with TGFβ or TβRI inhibitor in U373 cells. Proneural markers *BCAN* and *OLIG-2* were not expressed in U373 cells (data not shown).

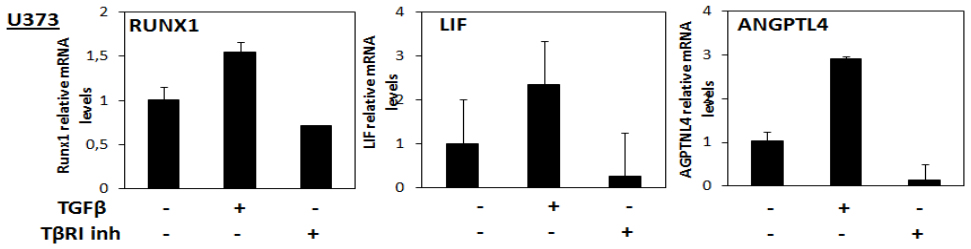


Figure 3.35. The TGFβ pathway regulates some of the mesenchymal genes. U373 glioma cells were treated for 7 days with 100pM TGFβ or 2μM of TβRI inhibitor or left untreated. Mesenchymal phenotype markers *RUNX1, LIF* and *ANGPTL-4* mRNA levels were measured by qRT-PCR. Data are presented as mean ± SD.

To study the role of Runx1 in this TGFβ-mediated mesenchymal transformation, we selected patient-derived neurospheres cultures with the highest levels of *RUNX1* to generate a stable knock-down of *RUNX1*. *RUNX1* knock-down decreased the levels of *LIF, PAI1, ANGPTL-4* and *YKL-40* mesenchymal phenotype markers and reduced the overall induction by TGFβ, suggesting that Runx1 could be a mediator of the mesenchymal trans-differentiation driven by TGFβ in GBM (Figure 3.36 A). We obtained similar results in other patient-derived neurospheres (GBM3, which also express high levels of *RUNX1*; data not shown) and in neurospheres derived from a patient, which was classified as mesenchymal by gene-expression clustering (GBM8). Interestingly in these GBM8 mesenchymal neurospheres, knock-down of *RUNX1* resulted in a decrease in the expression of several mesenchymal markers (*RUNX1*, *LIF* and *PAI1*) and also an increase in proneural markers (*BCAN* and *OLIG-2*) (Figure 3. 36 B). These results suggest that Runx1 is necessary for the mesenchymal phenotype in GBM samples.

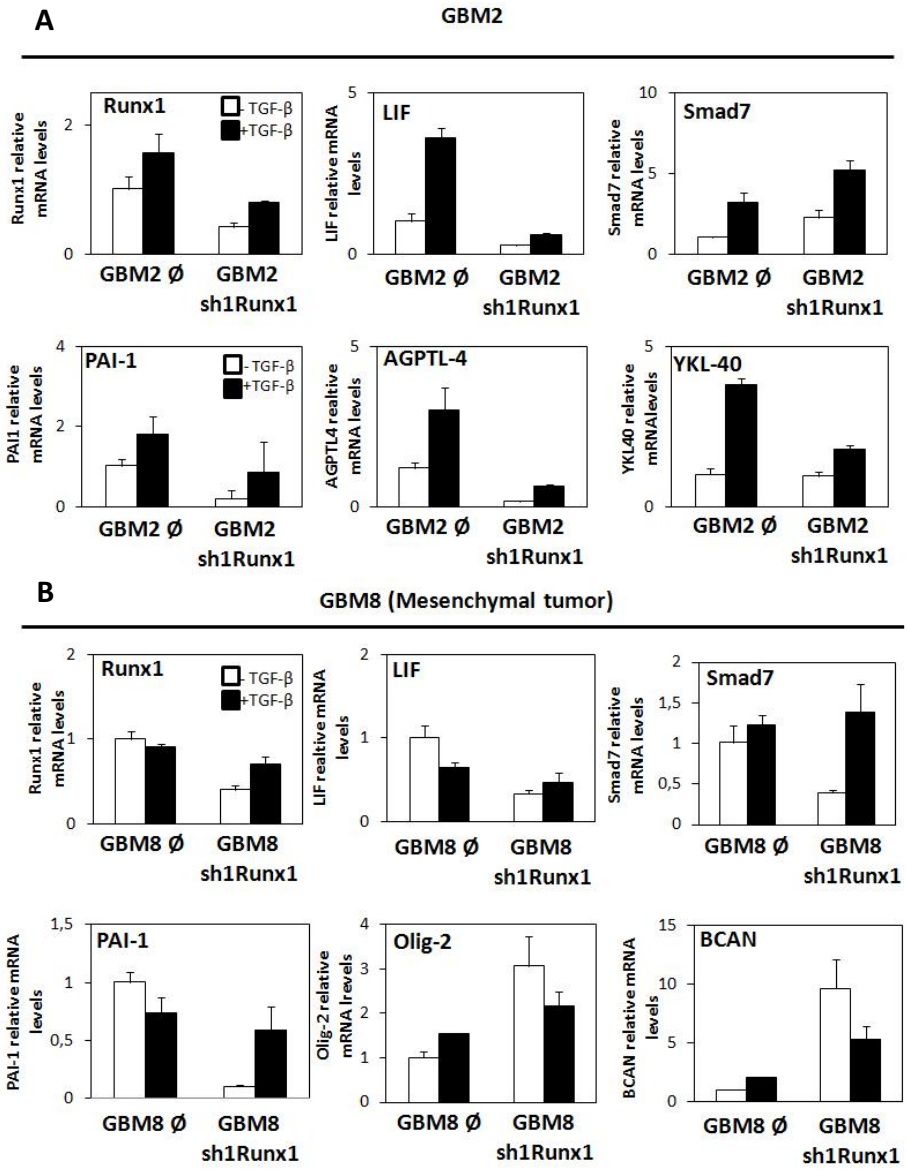


Figure 3.36. Runx1 is necessary for the mesenchymal phenotype of GBM. GBM2 and GBM8 neurospheres were infected with lentivirus with a short hairpin targeting *RUNX1*. Cells were treated for 7 days with 100pM TGFβ or left untreated. Mesenchymal markers *RUNX1, LIF, YKL-40* and *ANGPTL4* mRNA levels were measured by qRT-PCR. Proneural markers OLIG-2 and BCAN mRNA levels were also measured by qRT-PCR. *SMAD7* mRNA levels are shown as a control of TGFβ pathway activation. Data are presented as mean ± SD.

When we overexpressed *RUNX1* in neurospheres derived from a proneural tumor (GBM7), we observed an increase in the expression and especially in the induction by TGFβ of several mesenchymal markers: *RUNX1*, *LIF*, *PAI-1* and *ANGPTL-4* (Figure 3.37) but not *YKL-40* (data not shown). Interestingly, *RUNX1* overexpression also reduced the mRNA levels of proneural markers *BCAN* and *OLIG-2* (Figure 3.37) suggesting that Runx1 might be increasing the differentiation towards a mesenchymal phenotype and preventing the proneural phenotype.

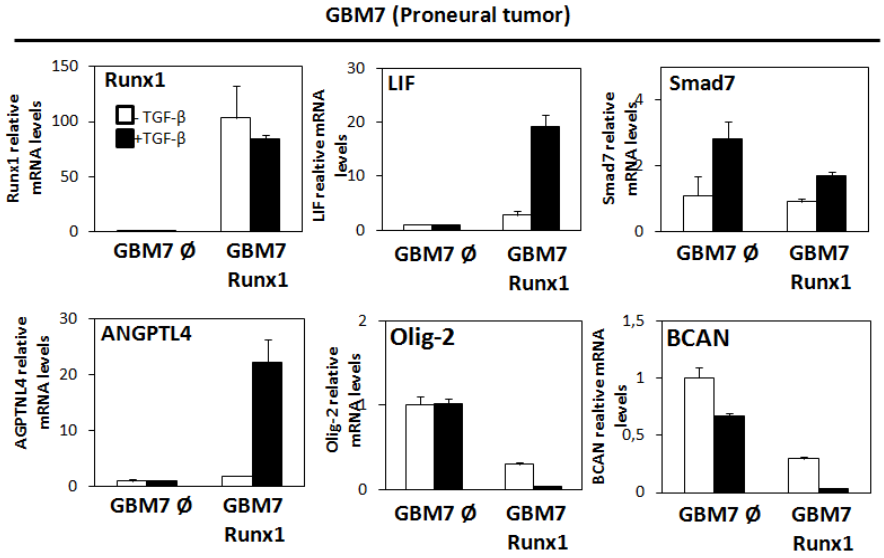


Figure 3.37. Overexpression of *RUNX1* in proneural-derived neurospheres. GBM7 neurospheres derived from a PN tumor were infected with lentivirus overexpressing *RUNX1*. Cells were treated for 7 days with 100pM TGFβ or left untreated. Mesenchymal markers *RUNX1*, *LIF*, and *ANGPTL4* mRNA levels were measured by qRT-PCR. Proneural markers *OLIG-2* and *BCAN* mRNA levels were also measured by qRT-PCR. *SMAD7* mRNA levels were measured as a control of TGFβ pathway activation. Data are presented as mean ± SD.

RUNX1 IS NECESSARY FOR TUMOR INITIATION *IN VIVO*

To further explore the role of Runx1 in GBM, we orthotopically inoculated patient-derived neurospheres with or without knock-down of Runx1 into immunocompromised mice and followed tumor progression. Survival was higher in mice harboring tumors with *RUNX1* knock-down than control tumors (Figure 3.38 A). We monitored tumor growth by MRI and we observed that at the time point when all control cells generated tumors, none of the cells with Runx1 knock-down generated any tumors in mice, although they eventually generated tumors, but those were smaller and with later onset (Figure 3.38 B). In an independent experiment, GBM2 derived neurospheres were infected with constitutively expressed luciferase and then *RUNX1* was knocked-down. Luciferase allowed us to follow and quantify tumor growth in an easy and non-invasive manner, using *in vivo* molecular image platform (IVIS- Xenogen). Cells with *RUNX1* knock-down generated significantly smaller tumors one month after inoculation (Figure 3.38 C and D). The differences, however, were reduced over the time (data not shown), suggesting that the role of Runx1 might be important on the tumor initiation and not on tumor progression.

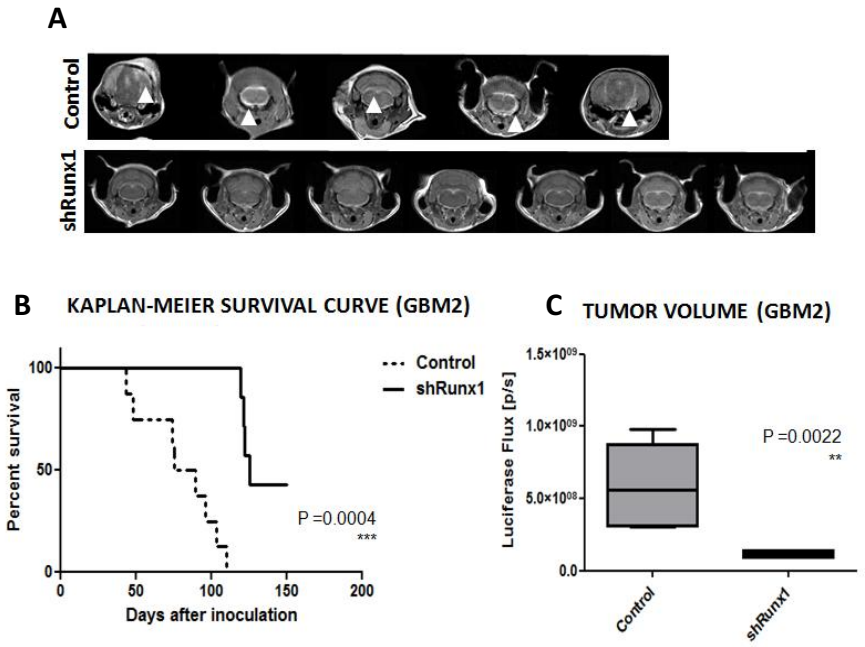


Figure 3.38. Runx1 is necessary for tumor initiation *in vivo.* **A.** GBM2 patient-derived neurospheres with or without *RUNX1* stable knock-down were orthotopically inoculated into NOD/SCID mice. MRI was performed 40 days after inoculation, when most of control neurosphere generated tumors. **B**. Kaplan-Meier survival curve showing the percent of survival of different both groups. Control mice died significantly earlier compared to mice inoculated with *RUNX1* knock-down neurospheres (p=0.0004). C. GBM2 patient-derived neurospheres constitutively expressing luciferase and with or without *RUNX1* knock-down were inoculated into NOD/SCID brains and tumor progression was followed by *in vivo* molecular imaging (IVIS). Luciferase total flux was quantified as a readout of tumor volume. Neurospheres with *RUNX1* knock-down generated significantly smaller tumors compared to normal cells (p value = 0.0022)

A careful histological study of the tumors confirmed a decrease in Runx1, which was not complete in all the cells, in tumors derived from neurospheres with a *RUNX1* knock-down, (Figure 3.39) and we also observed a reduction in LIF levels in Runx1 knock-down tumors and reduced expression of the mesenchymal marker YKL-40. In two cases we observed an increased expression of the proneural marker Olig-2 in tumors with Runx1 knock-down, suggesting a possible transformation from mesenchymal to proneural phenotype caused by the Runx1 knock-down.

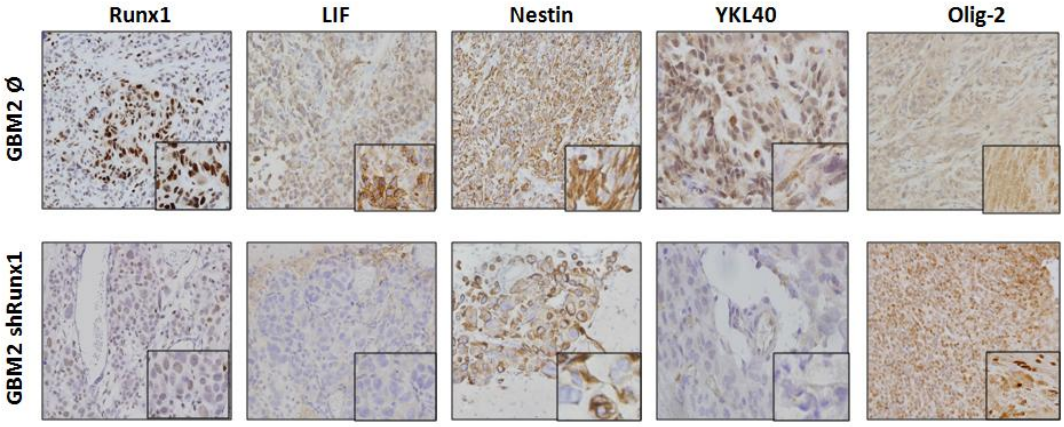


Figure 3.39. Histological analysis of patient-derived tumors**.** GBM2 patient-derived neurospheres with or without *RUNX1* stable knock-down were orthotopically inoculated into NOD/SCID mice. After sacrifice, we performed IHC of brain tumor sections staining for different markers: Runx1, LIF, Nestin, YKL-40 and Olig-2. Representative 10x images are shown with 20x magnification.

To confirm our findings, we FACS sorted human cells from mouse brain cells using MHC I antibody. We analyzed gene expression of sorted cells by quantitative RT-PCR. Although there was some variability in the different samples, we were able to observe a decrease in *RUNX1*, LIF, *ID1* and CD44

expression, and a decrease in *YKL-40* mesenchymal marker levels. Also we observed an increase in the expression of *OLIG-2* (Figure 3.40 A and B).

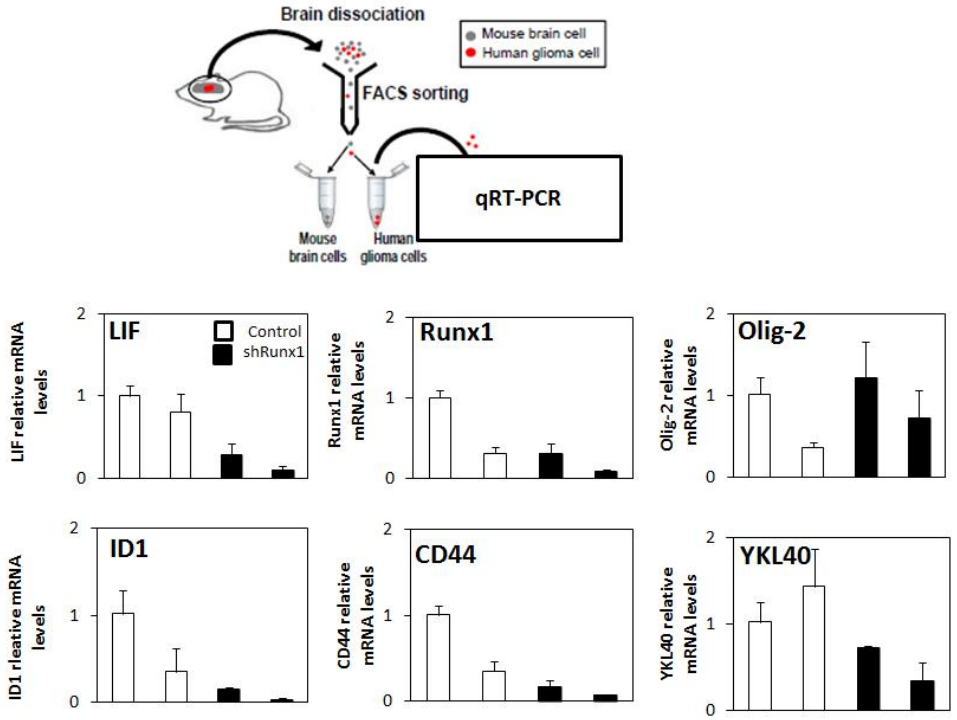


Figure 3.40. Characterization of gene-expression from patient-derived tumors with or without *RUNX1* knock-down. **A.** GBM2 patient-derived neurospheres with or without *RUNX1* stable knock-down were orthotopically inoculated into NOD/SCID mice. After sacrifice, human cells were separated from mouse brain by MHC class I sorting and qRT-PCR was performed. **B.** mRNA levels of *RUNX1*, *LIF, OLIG-2, ID1, CD44* and *YKL-40* measured by qRT-PCR. Data are presented as mean ± SD.

RUNX1 OVEREXPRESSION INCREASES *IN VIVO* TUMORIGENIC POTENTIAL OF PATIENT-DERIVED NEUROSPHERES

We overexpressed *RUNX1* in patient-derived neurospheres from a Proneural tumor that express lower levels of *RUNX1* compared to other neurospheres. We ectopically and constitutively expressed luciferase to be able to follow and quantify tumor growth. We inoculated those cells into NOD/SCID mice and we quantified tumor volume by *in vivo* molecular imaging using IVIS Xenogen Platform. Interestingly, *RUNX1* overexpressing neurospheres generated significantly bigger tumors one month after the inoculation, further supporting the important role of Runx1 in glioma progression (Figure 3.41).

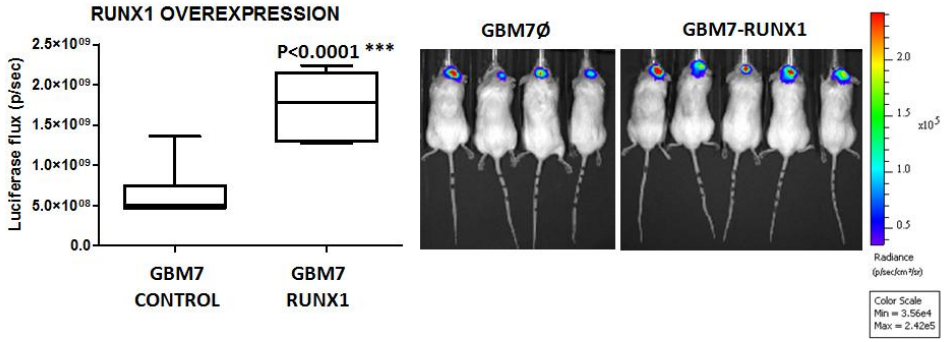


Figure 3.41. *RUNX1* overexpression increases *in vivo* tumorigenic potential of patient-derived neurospheres. GBM7 neurospheres were infected with lentivirus expressing luciferase and lentivirus expressing *RUNX1*. Control and Runx1 neurospheres were inoculated orthotopically into NOD/SCID mice. Tumor size was monitored by in vivo imaging of luciferase activity. One month after inoculation, *RUNX1* overexpressing neurospheres generated significantly bigger tumors compared to control neurospheres ($p<0.0001$). Right panels show luciferase activity measured by in vivo molecular imaging.

RUNX1 IS OVEREXPRESSED IN MALIGNANT GBM AND IT IS A POOR-PROGNOSTIC FACTOR IN GLIOMA PATIENTS

Using Oncomine database, we compared expression of Runx1 in normal brain and in different grades of glioma. In normal brain, Runx1 is only mildly expressed in the cytoplasm of some astrocytes or neurons. In glioma, Runx1 is significantly overexpressed by 4.359 fold with a p value of 1.59e-11. When comparing different grades of glioma, we found that *RUNX1* was significantly overexpressed by 2.63 fold in GBM (grade IV malignant glioma) compared to lower grade glioma (p value 4.06e-9) (Figure 3.42 A and B).

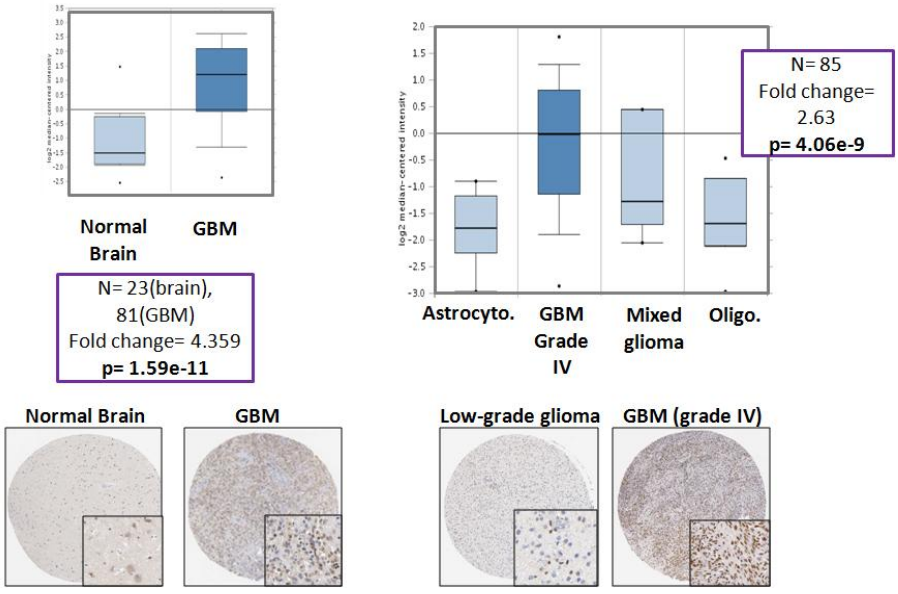


Figure 3.42. A. *RUNX1* expression levels in 23 normal brain samples and 81 GBM samples (Data from Oncomine). Lower panels: representative section of normal brain and GBM were stained with Runx1 for IHC. **B.** *RUNX1* expression levels of 85 different graded glioma. Lower panels: representative sections of low-grade glioma dn high-grade glioma (GBM) were stained for Runx1 IHC. Representative 10x images are shown with 20x magnification.

When we analyzed survival of GBM patients using the REMBRANDT database, we found that patients with Runx1 up-regulation of 10 fold have poor overall survival (Figure 3.43). This demonstrates that Runx1 is a poor-prognostic factor for glioma patients, consistent with its role as a mediator of the TGFβ oncogenic effect that we have described.

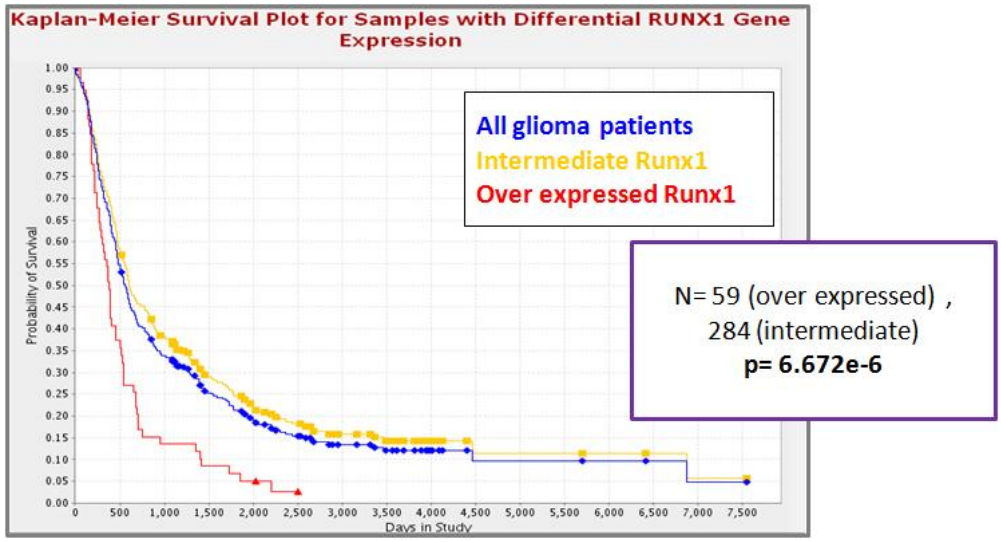


Figure 3.43. Kaplan-Meier survival plot for 343 glioma patients. 59 patients have overexpression of *RUNX1* (10 fold) and 284 intermediate expression of RUNX1. Patients with *RUNX1* overexpression showed a significant decrease in overall survival (p=6.673e-6).

DISCUSSION

GBM AND FAILURE OF CURRENT THERAPIES

Glioblastoma is one of the most deadly types of tumors, with a median overall survival of only 15 months despite of the treatment (Stupp, Mason et al. 2005). The standard of care therapy consists in surgical resection combined with chemo- and radiotherapy. Nowadays, the survival of GBM patients is correlated with the extent of the resection, which depends on many factors such as location of the tumor and the physical status of the patient before the surgery (what is known as the Karnofsky Performance Status, giving a certain score for each the patient, an index that takes into account the age and sex of the patient as well as their physical capacities (Yates, Chalmer et al. 1980; Balducci, Fiorentino et al. 2012).

Although there have been recent advances in understanding the biology and progression of GBM, those findings have not yet translated into a significant improvement in patient care or survival. There is a need for finding new therapeutic approaches to help increase the survival of GBM patients.

Many advances have been made in understanding the pathways that are deregulated in GBM, such as PI3K, p53 and Ras/MAPK. Although their role GBM progression is clear, clinical trials targeting those pathways did not show any success. Hence, there is a need to discover new therapeutic targets that could improve GBM patients care.

In this project I have been focused on the study of the TGFβ pathway and its oncogenic role in GBM. TGFβ is a cytokine which is important in embryonic development and tissue homeostasis (Massague 2012). TGFβ has a dual role: it typically acts as a tumor suppressor inhibiting proliferation and inducing cell cycle arrest. But in many different tumor

types including most carcinomas, TGFβ has an oncogenic role, promoting proliferation, epithelial-to-mesenchymal transition, metastasis, angiogenesis and immune suppression (Massague 2008; Heldin, Vanlandewijck et al. 2012). It has been recently described by our group and others (Bruna, Darken et al. 2007; Penuelas, Anido et al. 2009; Joseph, Balasubramaniyan et al. 2013) that TGFβ has an important oncogenic role in glioma, such that patients with a hyperactive TGFβ pathway have a poor prognosis.

In our group, we have described how TGFβ increases glioma progression through the induction of *PDGFB*. Furthermore, TGFβ is involved in GIC self-renewal and maintenance making it a very promising therapeutic target. (Ikushima, Todo et al. 2009; Penuelas, Anido et al. 2009; Seoane 2009). In our group we have demonstrated that the TGFβ oncogenic role on GIC self-renewal is mainly through the secretion of LIF and subsequent activation of JAK-STAT3 pathway (Penuelas, Anido et al. 2009).

This population of GICs shares some characteristics with normal stem cells, such as asymmetric division, self-renewal capacity and pluripotency. This cells have the capacity to initiate the tumor and, furthermore, are resistant to conventional therapies such as chemo- or radiotherapy (Rich 2007; Chen, Nishimura et al. 2010; Scheel, Eaton et al. 2011; Chen, Li et al. 2012; Chesler, Berger et al. 2012). Understanding the biology of this cell population would lead us to develop specific therapies targeting GICs thus decreasing the probability of relapse after treatment and improving the survival of patients. There are many pathways that have been extensively studied regarding Cancer Initiating Cells in general. The most typical ones are: Notch, Sonic-Hedgehog (Shh) and Wnt, which are important during

normal embryonic development and have a relevant role in normal stem cell biology.

A TRANSLATIONAL RESEARCH APPROACH

In order to translate the new findings and targets discovered in the laboratory into clinical practice, we use a model based on translational research. To do so, we generate human-derived tumors in immunocompromized mice in order to screen different drugs that are being tested in clinical trials. With this, we want to study the mechanisms of response of each tumor or tumoral subpopulations to targeted therapies. Due to our close collaboration with the neurosurgery department which provides us with fresh tumor samples, we are generating a collection of patient-derived neurosphere cultures with matched DNA and RNA samples taken from the patient. We are inoculating these patient-derived neurospheres into immunocompromized mice systematically, and we are able to generate a tumor with similar characteristics to the patient's at the level of histology, expression of biomarkers, location within the brain and response to different treatments. Furthermore, we recently started to sequence the whole cancer genome of the tumor cells using High Throughput Genome Sequencing and we are comparing the mutations and copy number aberrations found in each patient with the tumor generated in the mouse model. Interestingly this is showing that our xenograft mouse model recapitulates very well the heterogeneity found in the patient's tumor and

we can test diverse therapies that are currently in clinical trials on animals to better understand how they respond.

As we are interested in targeting GICs and we know that the TGFβ pathway is important for its maintenance, we used the TβRI inhibitor LY 2109761 to study the consequences of TGFβ pathway inhibition in GICs. Intially, we observed that *ID1* and *ID3* expression were significantly decreased after treatment with TβRI inhibitor in a microarray generated from 11 patient-derived samples that were treated with TβRI inhibitor. Id proteins are basic Helix-Loop-Helix proteins, known as inhibitors of differentiation. They typically interact with E-proteins preventing its binding to the DNA. Id proteins have a role in cell growth, differentiation and senescence. *ID1* is expressed in B1 neural progenitors in adult brain (Nam and Benezra 2009) and is known to be related with different types of cancers and cancer initiating cells (Benezra, Davis et al. 1990; Ruzinova and Benezra 2003; Perk, Iavarone et al. 2005; Gupta, Perk et al. 2007; Niola, Zhao et al. 2012). Interestingly, Id proteins were initially described to be inhibited by TGFβ and induced by BMP signaling. In our work, we characterize the molecular mechanism of *ID1* induction by TGFβ and we found that TGFβ and BMP are binding in the same region of *ID1* promoter. However, while TGFβ is acting as a repressor in epithelial cells, it activates expression of ID1 in GBM neurospheres. The *ID1* repression is mediated by ATF3, which in normal epithelial cells cooperates with Smads and inhibit the transcription of *ID1*. GBM neurospheres do not induce *ATF3*, and then Smads are capable to activate *ID1* transcription, probably by cooperating with other transcription factors that act as activators. This shows the importance of interacting cofactors and cellular context in determining the differential gene responses of TGFβ.

We also focused our attention in CD44^{high}, because it has been described to be a marker for some CICs in leukemia and some solid tumors such as breast and colon carcinoma (Jin, Hope et al. 2006; Mani, Guo et al. 2008; Bellizzi, Sebastian et al. 2013). We found a correlation between CD44^{high} and Id1 positive cells, although CD44 was more broadly expressed in GBM samples. Our hypothesis is that only those cells that are both positive for Id1 and express high levels of CD44, have the capacity to initiate tumors, and thus can be defined as GICs.

We demonstrated that this population of CD44^{high}/Id1 positive cells has indeed tumor initiating capacity by performing *in vivo* limiting dilution assays. We were able to generate tumors in immunocompromized mice with only 1,000 CD44^{high} cells, whereas CD44^{low} cells were less efficient in generating tumors *in vivo*, demonstrating that the CD44^{high} population have cancer initiating capacity. This is particularly important because one of the main problems in the field of CIC research is the lack of reliable, physiological markers. Due to high heterogeneity between different patients and within the same tumor, different groups have postulated different biomarkers to isolate this population of GICs, such as CD133, SEEA-1 or ALDH1+ (Singh, Clarke et al. 2003; Son, Woolard et al. 2009; Ma, Ma et al. 2013). It is critical to have a reliable marker to isolate and then study this population of GICs and its biology in order to develop targeted therapies. Here we demonstrated that CD44^{high}/Id1 + are markers for GICs, by functionally characterizing this population. Since the publication of our work, other groups have used CD44^{high} as a marker for GICs, and furthermore, have demonstrated that it plays an important role in tumorigenesis, being necessary for cell migration and invasion (Yoshida, Matsuda et al. 2012; Zhao, Damerow et al. 2012; Piao, Wang et al. 2013). The most clinically relevant finding of this work is that we demonstrated

that by inhibiting the TGFβ pathway we can target CD44^{high}/Id1 + GICs *in vitro* and *in vivo*. Furthermore, we validated these results in a human patient sample that was treated with the TβRI inhibitor in a phase I clinical trial. One of the main causes of death in GBM patients is recurrence after treatment. Because GICs are responsible for tumor reinitiation, we hypothesized that treatment with TβRI inhibitor would prevent this recurrence. We were able to demonstrate this in our *in vivo* xenograft model of GBM, by re-inoculating human tumoral sorted cells into new recipient mouse, in an *in vivo* model that mimics recurrence after complete tumor resection. When we orally treated mice with TβRI inhibitor, the population of CD44^{high}/Id1 + cells decreased, and the resulting cells were less able to re-initiate tumors *in vivo*.

It has been recently published that Id1+ cells correlate with higher self-renewal capacity but not with tumor growth potential in high-grade glioma mouse models (Barrett, Granot et al. 2012). Authors describe that Id1^{high} glioma cells have stem-cell characteristics such as self-renewal capacity and expression of stem-cell markers Prominin-1 and Id3, whereas Id1^{low} have limited self-renewal capacity but higher proliferative potential associated with expression of Olig-2, which is known to regulate proliferation in normal neural progenitors (Ligon, Huillard et al. 2007). In contrast with our experiments, authors postulate that Id1^{low} cells are more efficient in initiating tumors compared to Id1^{high}. This controversy might be explained by the use of different *in vivo* mouse models (GEMM vs patient-derived xenograft) and by the fact that when they are sorting Id1^{high} cells, they might be also selecting normal enodothelial cells which are abundant and express high levels of Id1, thus diluting the amount of GICs.

In our work we not only demonstrate the importance of this population of $CD44^{high}$/Id1 + having GIC capacity, but also the role of the TGFβ pathway in maintaining this population.

The role of the TGFβ pathway in regulating CICs is not limited only to glioma. In many other tumor types, CICs are described to be regulated by TGFβ or other family members. Of note, it has been recently described that TGFβ increases breast CICs in claudin-low patients (Bruna, Greenwood et al. 2012). Activin, another member of the TGFβ super-family which also signals through the phosphorilation of Smad2/3, has been shown to be related with an increase of CIC and self-renewal capacity in some cancers such as pancreatic cancer and melanoma (Topczewska, Postovit et al. 2006; Postovit, Seftor et al. 2007; Lonardo, Hermann et al. 2011; Strizzi, Hardy et al. 2011). This reveals the universality of TGFβ as a regulator of CICs in different tumor types.

ENDOTHELIAL CELLS SECRETE TGFβ CREATING A PERIVASCULAR NICHE NECESSARY TO MAINTAIN GICS

The importance of tumor microenvironment and the relationship between tumor cells and surrounding cells has been extensively revised (Hu and Polyak 2008; Polyak, Haviv et al. 2009; Barcellos-Hoff, Lyden et al. 2013). TGFβ has well documented role in tumor microenvironment in different cancer types, being the mediator of the interactions between cancer cells and their niche. TGFβ can be secreted by both, tumor cells or stroma/microenvironment cells in a finely regulated balance (Stover, Bierie et al. 2007).

But in glioma patients, it is not clear which cells produce the abnormally high levels of TGFβ. Our group has demonstrated that TGFβ can be secreted by tumoral cells, in an autocrine loop that produces aberrantly high levels of TGFβ in some GBM patients (unpublished data).

Now we also demonstrate that tumor endothelial cells secrete TGFβ to the microenvironment. This is in concordance with what was previously described that GICs tend to be located in the proximity of tumor blood vessels where they have an appropriate microenvironment with the presence of specific growth factors they require (Calabrese, Poppleton et al. 2007; Gilbertson and Rich 2007; Charles, Holland et al. 2011; Heddleston, Hitomi et al. 2011). We observed that in some GBM patients, $CD44^{high}$ and Id1+ GICs tend to be located in close proximity to tumor blood vessels. That led us to think that endothelial cells could be secreting cytokines or growth factors required for maintenance of GICs. Since we have previously demonstrated the requirement of TGFβ for maintenance of GIC self-renewal capacity, we investigated whether TGFβ was present in the perivascular niche and was secreted by endothelial cells. We showed that endothelial cells provide TGFβ to the GICs that are located in its proximity and that TGFβ is important for maintaining their characteristics such as self-renewal capacity and tumor formation. To prove that endothelial cells secrete factors required by GICs, we pre-conditioned the media for 72 hours with endothelial cells, and after filtering the media, we added to different patient-derived neurospheres cultures. We observed that endothelial-cell pre-conditioned media increases the $CD44^{high}$/Id1 + population, self-renewal capacity and tumorigenic potential of patient-derived neurospheres and this can be reverted by treatment with anti-TGFβ blocking antibodies or the TβRI inhibitor, demonstrating that TGFβ was necessary for this effect. Interestingly, we consistently observed an

induction in *ID1* expression, to a higher level than that observed when we treat with recombinant TGFβ alone. This led to the speculation that there are additional growth factors or cytokines present in endothelial cell pre-conditioned media that cooperate with TGFβ in the induction of *ID1*. Preliminary results from our lab suggest that there may be cooperation between some BMP family members and TGFβ in the induction of *ID1*. Further experiments are needed to address this question.

Some authors describe that, besides the perivascular niche for GICs, there are also some GICs residing in hypoxic niches, far from tumor vessels and with low oxygen concentration. In our GBM samples we do indeed observe small groups of $CD44^{high}$/Id1 + cells although they are not as predominant as the perivascular ones. We believe that GICs can reside near tumor blood vessels, but also in hypoxic niches. We might find both types of GICs maybe within the same patient. What remains unknown is whether these GICs that reside in a perivascular niche and the ones that reside in the hypoxic niche are the same entity or they are two distinct entities both with tumor initiating capacity.

Some authors postulate that the intimate interplay between cancer cells and surrounding niche is a crucial determinant of cancer growth, even from the early stages. For this reason, cancer niches can be considered potential targets for cancer prevention and therapy (Barcellos-Hoff, Lyden et al. 2013). Thus, targeting the perivascular niche for GICs by using inhibitors of the TGFβ pathway may be an effective therapy for GBM patients.

TARGETING GICS

GICs are typically resistant to conventional therapies, such as radio- and chemotherapy (Rich 2007; Izumiya, Kabashima et al. 2012). Because of their resistance and their tumor initiating capacity, GICs are responsible of relapse after treatment which usually causes the death of the patient. It is of utmost importance to develop new therapeutic approaches to eradicate them. During the past few years, GICs have been extensively studied, and we are starting to understand the pathways important for their biology thus providing potential targets for pharmacological invervention. For example, it is known the importance of Notch pathway for Cancer Initiating Cells (Bolos, Blanco et al. 2009). There are several inhibitors of the Notch pathway, mostly inhibitors of ɣ-secretase, which is the enzyme that performs the cleavage of Notch, releasing the intracellular domain and triggering the pathway activation. Of note, inhibition of the Notch pathway by ɣ-secretase inhibitors may increase the radiosensitivity of CICs (Wang, Wakeman et al. 2010), suggesting that therapies targeting CICs may have an additional benefit in combination with standard of care chemo- and radiotherapy.

Here we have studied the TGFβ pathway and its oncogenic role, focusing mainly on the maintenance of GICs (Seoane 2009; Anido, Saez-Borderias et al. 2010). Due to the relevant role of the TGFβ pathway in GICs, there are several TGFβ inhibitors being developed for the treatment of glioma.

DEVELOPMENT OF TGFβ INHIBITORS IN THE CLINIC

As extensively discussed, the TGFβ pathway plays an intriguingly dual role in terms of cancer development and it has a very relevant role in many

human diseases (Blobe, Schiemann et al. 2000). Importantly, in many advanced carcinomas as well as in Glioma, TGFβ has an oncogenic role and is a poor-prognosis factor. Thus, many pharmaceutical companies have been working on developing different strategies to block activation of this pathway. There are three different ways to inhibit the TGFβ pathway: firstly, inhibit TGFβ mRNA expression by using anti-sense RNA; secondly, inhibit the binding of the ligand to its receptor by using blocking antibodies; and finally, inhibiting activity of the TGFβ receptor by blocking ATP binding and thus shutting down pathway activation. There are several different compounds currently in clinical trials. In the first group of compounds, we can mention Trabedersen (AP-12009) is a TGFβ2 anti-sense mRNA from Antisense Pharma that has recently completed a phase I/II clinical trial in high-grade recurrent or refractory glioma patients (NCT00844064, NCT00431561). Interestingly, although the clinical trial was originally designed to evaluate safety and toxicity, prolonged survival compared to literature data was observed in some patients. Pre-clinical and some clinical data showed promising results, implicating that targeting TGFβ2 in those patients with malignant glioma or other highly malignant tumors with elevated TGFβ2 levels (Hau, Jachimczak et al. 2007; Bogdahn, Hau et al. 2011). The same pharmaceutical company is developing an anti-sense oligonucleotide targeting TGFβ1, called AP-11014, which is currently undergoing pre-clinical studies in models of lung, colon and prostate cancer (K.-H. Schlingensiepen et al 2004). Using the second strategy to inhibit the TGFβ pathway activity - blocking the binding of cytokine to its receptor - GC1008 is an anti-TGFβ monoclonal antibody developed by Genzyme that is currently being tested for the treatment of melanoma, renal cell carcinoma and pulmonary fibrosis (NCT00356460, NCT00923169). This antibody is well tolerated and neutralization of TGFβ

holds promise as a novel cancer therapy (J. C. Morris, et al 2008). There is also a novel antibody targeting the TGFβ Type II Receptor (IMC-TR1) from ImClone/Eli-Lilly. After showing promising results in pre-clinical studies, delaying tumor growth and metastasis, it is now undergoing Phase I clinical trials in breast and colon cancer patients (NCT01646203) (Zhong, Carroll et al. 2010).

In this project, we benefited from collaboration with the pharmaceutical company Eli-Lilly which provided us with a newly developed small-molecule inhibitor of the TGFβ type I Receptor: LY2109761 and its derivative LY215799. These molecules represent the third of the strategies to inhibit the TGFβ pathway and are undergoing clinical trials for different types of cancer. Our hospital is actively participating in some of the clinical trials, performing for example an initial dose-escalation trial for recurrent glioblastoma patients (NCT01682187). Early phase clinical trials showed promising results with some of the patients having a partial response according to Response Evaluation Criteria in Solid Tumors (RECIST) (Therasse, Arbuck et al. 2000). Some clinical trials are also being conducted to determine the efficacy of this drug in combination with chemo- and radiotherapy as a first-line treatment in recurrent glioblastoma patients (NCT01582269 and NCT01220271). Additionally, this compound is being tested in hepatocellular carcinoma (NCT01246986) and in metastatic advanced or unresectable pancreatic cancer (NCT01373164). Our privileged position in a research institution that works in close collaboration with clinicians gives us the opportunity to obtain tumor samples as well as blood samples, and follow the progress of these clinical trials, so we can translate our findings in the laboratory directly back to clinical management and conversely, the clinical data can help us develop better models to study the effect of the inhibitors. The integration of a

multidisciplinary team composed of surgeons, oncologists, research scientists, and pharmaceutical companies is the best combination to better understand the biology of this disease, and what drives the response to certain compounds in certain patients and not in others. We are optimistic that this approach will help to improve the treatment of this dismal disease.

It is becoming clear that stratification of patients involved in clinical trials essential: inter- and intra-tumoral heterogeneity of tumors means that not all patients will respond to the same compound. Thus the need to better understand the molecular characteristics that drive the response to a certain drug in order to better design clinical trials that would translate into benefits for the patients. Our role as a translational laboratory is to study the molecular mechanisms of response to certain pathway inhibition both *in vitro* and *in vivo*, to provide this kind of information that could help to design better therapies and clinical trials.

COMBINATION OF TβRI INHIBITOR AND RADIOTHERAPY TO PREVENT RECURRENCE

In our group we are particularly interested in the study of GICs because they are usually responsible for resistance to current treatments and therapeutic failure. It has been described that CICs are generally resistant to radiotherapy due to its preferential activation of DNA-repair pathways (Bao, Wu et al. 2006; Rich 2007). This could explain the high index of recurrence in GBM patients after treatment with ɣ-radiation. CICs are also more resistant to conventional chemotherapeutic agents (Rich and Bao 2007; Tanei, Morimoto et al. 2009; Abubaker, Latifi et al. 2013). We

explored the resistance of GICs CD44^{high}/Id1+ to radiotherapy using our *in vivo* xenograft mouse model that recapitulates the characteristics of the patient's tumor. We observed a significant increase in the CD44^{high}/Id1+ population both *in vitro* and *in vivo* showing that the GICs are radioresistant as was previously reported for CICs (Bao, Wu et al. 2006; Rich 2007). It is also described that ɣ -radiation can induce the expression of TGFβ by stroma cells, although the mechanism remains unclear (Martin, Vozenin et al. 1997; Dancea, Shareef et al. 2009). We then postulated that this could be a mechanism to protect GICs against irradiation, as TGFβ is has a relevant role in maintaining the GIC population. We demonstrated that treatment with TβRI inhibitor in combination with irradiation decreases the CD44^{high} population of GICs in different patient-derived samples. Similar results were obtained by others (Zhang, Kleber et al. 2011; Hardee, Marciscano et al. 2012). In their work they combined ɣ-irradiation with inhibition of the TGFβ pathway using the same TβRI inhibitor that we are using, the small molecule LY2109761. They observed that combination of TβRI inhibitor with radiotherapy decreased the percentage of CSCs *in vitro* and prolonged survival *in vivo.* Interestingly, they found that the tumors had less mesenchymal characteristics and decreased angiogenesis after the combination of both treatments.

These results suggest that combination of TβRI inhibitor with standard of care therapy (radio- and chemotherapy) in the treatment of glioma would be a promising approach. This combination therapy is currently in clinical trials in our hospital for GBM patients. A phase I clinical trial was concluded with satisfactory results and some patients showed a partial response during the trial. Now, they are recruiting more patients to undergo a phase II clinical trial in which the TβRI inhibitor would be a first-line treatment combined with radio- and chemotherapy. Our results suggest that this

combination might be more effective as TβRI targets the GIC population responsible for tumor re-initiation after conventional treatment.

To confirm in the laboratory that combining radiotherapy and the TβRI inhibitor can efficiently decrease tumor relapse, we are planning to re-inoculate tumoral sorted cells from animals which were irradiated and concomitantly treated with TβRI inhibitor *in vivo* into new immunocompromised mice. With this approach, we are mimicking a relapse after a complete tumor resection followed by radiation therapy, which is what happens in most of GBM patients. This preclinical model of relapse of GBM is very useful to test combinations of different treatments that are currently given in the clinical practice.

RUNX1 AS A KEY MEDIATOR OF TGFβ ONCOGENIC EFFECT IN GLIOMA

The Runx family of transcription factors has been related to several types of cancers, and they have been especially studied in leukemia. One notable characteristic of these transcription factors is that they can act as oncogenes or tumor suppressors, depending on their bound cofactors and the recruited complex of co-activators and co-repressors. Thus, as for TGFβ, Runx1 has also a dual role in cancer, depending on the cellular context, surrounding microenvironment and epigenetic modifications.

In late 2010, Runx1 was identified as one of the six transcription factors that regulate the mesenchymal subclass of GBM, which is the one with worst prognosis (Phillips, Kharbanda et al. 2006; Carro, Lim et al. 2010). They used a bioinformatics approach to study the promoter regions of all

genes up-regulated in the mesenchymal subtype, to find common putative transcription factor binding sites. This study reveals a transcriptional module that activates the expression of mesenchymal genes in malignant glioma. The six transcription factors that regulate the mesenchymal phenotype in glioma are *RUNX1*, C/EBP-beta, STAT3, b-HLHB2 and FOSL2, as transcriptional activators, and ZNF238 possibly acting as a repressor. These results show that activation of this small regulatory module is necessary and sufficient to initiate and maintain the mesenchymal phenotypic state in cancer cells, which correlates with poor clinical outcome. These results are very interesting for our project, as we observe that Runx1 is important for maintaining this mesenchymal phenotype in glioma-derived neurospheres. Interestingly, many of the genes up-regulated in these mesenchymal tumors are also genes regulated by TGFβ. We speculated that TGFβ might be driving this mesenchymal transformation, as it does in epithelial cells as a typical and potent inducer of EMT. While we cannot talk about an EMT processes in gliomagenesis, we can speculate that there may be similar underlying processes and pathways involved in mesenchymal transition in GBM.

We have focused our interest on those genes from the mesenchymal signature that are known to be regulated by TGFβ such as *LIF*, *SERPINE1* or *ANGPTL-4*. We hypothesized that Runx1 was necessary for TGFβ-induced mesenchymal transformation in glioma. Our results indicate that some of the TGFβ-regulated genes in the mesenchymal signature (*YKL-40*, *ANGPTL-4*, *SERPINE1*, *LIF*, *CD44*) are decreased and no longer induced when we knocked-down *RUNX1* in neurospheres, pointing out the importance of this transcription factor in the TGFβ-mediated mesenchymal transformation. We have some indication that this also occurs *in vivo* as when we knock-down Runx1 in GBM-derived neurospheres, those cells were less able to

generate a tumor in immunocompromized mice and tumors had lower levels of some mesenchymal markers such as LIF and YKL-40 and increased expression of the proneural marker Olig-2 in some areas of the tumor. We want to further elucidate the role of Runx1 as an important mediator of the mesenchymal phenotype *in vivo*. To do so, we have knocked-down *RUNX1* in patient-derived neurospheres and we are currently inoculating them and following the tumor progression. We will isolate human tumor cells by FACS-sorting and analyze the gene-expression profile of tumors with *RUNX1* knock-down and compare them with control tumors. This will help confirm our *in vitro* results that Runx1 is an important mediator of mesenchymal phenotype also *in vivo.*

Interestingly, it has been recently postulated that one of the mechanisms by which TGFβ increases the CIC population is the induction of EMT process. Some authors believe that EMT, not only generates more motile cells with higher invasive capacity, but also generates cells with tumor-initiating capacity (Brabletz, Jung et al. 2005; Mani, Guo et al. 2008; Morel, Lievre et al. 2008; Polyak and Weinberg 2009; Scheel, Eaton et al. 2011). This important link between TGFβ as an inducer of EMT and CICs suggest that similar mechanisms could be governing the mesenchymal transdifferentiation in GBM tumors.

Our results also show that Runx1 has an important role in maintaining the GICs population. When we decreased Runx1 levels, we observed a consistent decrease in *ID1* induction by TGFβ and a decrease in $CD44^{high}$ population. Several authors postulate that CSCs might be derived from an EMT process (Mani, Guo et al. 2008; Singh and Settleman 2010). In breast, TGFβ induced EMT generates cells with characteristics of CSCs, such as the $CD44^{high}/CD24^{low}$ population. Similarly, in mesenchymal tumors we find

broader expression of CD44 and typically a higher proportion of $CD44^{high}$ population. We postulate that, as Runx1 is a key mediator of mesenchymal subclass and mesenchymal transformation might be responsible to generate $CD44^{high}$/Id1+ GICs, it seems logical to think that it is also has a role to maintain the $CD44^{high}$/Id1+ population of GICs. This has further implications as GICs are critical targets for therapeutic approach. Our results demonstrated that Runx1 is involved in maintaining this population of $CD44^{high}$/Id1+ postive GICs, as a decrease in *RUNX1* expression leads to a decrease in the GIC population. Runx1 is also necessary to maintain the characteristics of GICs such as self-renewal capacity and stemness. Knock-down of *RUNX1* causes a decrease in the expression of stem markers such as *NESTIN* or *SOX2*, and caused an increase in the expression of GFAP, an astrocytic differentiation marker. This result suggests that Runx1 prevents the differentiation of GICs towards an astrocytic lineage. No significant changes were observed in the expression of other differentiation markers such as O4 (oligodendrocitic marker) or Tuj1 (neuronal marker). This differentiation phenotype is similar to a previous result from our group (Penuelas, Anido et al. 2009), where LIF is found to increase the expression of stem markers (*NESTIN*, *SOX2* and *MUSASHI-1*) and decrease the expression of differentiation markers (*GFAP*, *O4* and *TUJ1*). As Runx1 is necessary for LIF induction, we attempted to rescue the effect of *RUNX1* knock-down by treatment with recombinant LIF. Intestingly, not all the effects of *RUNX1* knock-down on regulating mesenchymal and stem markers were restored by LIF treatment. *RUNX1* knock-down caused a decrease in the expression of the stem markers *NESTIN* and *SOX2*, which was not rescued after treatment with recombinant LIF. On the other side, *RUNX1* knock-down caused an increase in *GFAP* expression which, in this case, was prevented by treatment with recombinant LIF. Similar results

were obtained when studying mesenchymal markers such as *CD44* or *ANGPTL-4*: *RUNX1* knock-down decreased the expression and induction by TGFβ of those mesenchymal markers, and treatment with recombinant LIF was not able to restore their levels. This result suggests that the critical role of Runx1 in maintaining GIC stemness capacity and mesenchymal phenotype is at least partly independent of LIF secretion.

At the beginning of this project, we were focused on understanding the molecular mechanism of *LIF* induction by TGFβ. As we find a Runx1 binding site near the Smad Binding Element, and Runx1 is a known co-factor that binds to Smads in the induction of certain genes (Hanai, Chen et al. 1999; Zhang and Derynck 2000), we postulated that Runx1 might be important for LIF induction. We clearly demonstrated this at many levels; first of all using an *in vitro* approach, with luciferase reporter assays and site directed mutagenesis to prove the importance of Runx1 in LIF induction by TGFβ. As this is an artificial system and we were overexpressing the constructs, we wanted to study the role of endogenous Runx1, and we knocked-down or overexpressed *RUNX1* and confirmed that *LIF* induction by TGFβ was decreased with *RUNX1* knock-down and increased with *RUNX1* overexpession. Interestingly, we were able to validate our results in glioma cell lines and also in patient-derived neurospheres. In all the patient-derived neurospheres that we have tested, we found similar results, suggesting the universality of this mechanism by which Runx1 is needed for TGFβ induction of LIF. However, when we further explored the role of Runx1 on other TGFβ targets and on mesenchymal signature genes, we found out that Runx1 has also an important role as a mediator of the TGFβ gene-expression response. In those tumors with a mesenchymal gene-expression signature and with high TGFβ activity, *RUNX1* knock-down decreased the induction of some mesenchymal TGFβ target genes. As

those tumors are characterized by a hyperactive TGFβ pathway, we postulate that those tumors will show an increased response to TGFβ inhibition as they are highly dependent on TGFβ activity. Thus Runx1 could be a biomarker for tumors with high TGFβ pathway activity and a mesenchymal phenotype that may respond better to TβRI inhibitors. Further *in vivo* experiments are needed in order to confirm this hypothesis.

Runx1 has been shown to have an oncogenic role in several cancer types, but this is the first time that we postulate that Runx1 acts as an oncogene in glioma. In glioma patients, Runx1 is more overexpressed in high-grade tumors compared to low-grade tumors and it is a poor-prognosis factor: higher expression of Runx1 correlates with lower overall survival.

We have been studying the role of Runx1 as a mediator of the induction of many different TGFβ targets. Interestingly, some of those TGFβ target genes that are regulated by Runx1 are molecules related to the immune system and inflammation, such as IL6 and LIF. One might hypothesize that Runx1 could have an important role in inflammation or modulation of the immune system. In our *in vivo* mouse model, we used immunocompromised mice to generate tumors derived from human samples and thereby avoid inter-species rejection. In this model, we are unable to study the role of the TGFβ pathway in modulating the immune system. Some targets of the TGFβ pathway related to the immune system and inflammation are affected by knock-down or overexpression of *RUNX1*, suggesting a possible role for Runx1 as a mediator of TGFβ immune suppression or tumor escape in cancer. Taking into account the importance of TGFβ modulating the immune system in tumors, we should further explore the role of Runx1 using different mouse models with a functional immune system. Since we cannot work with patient-derived

samples in immune-competent mice, one option is the use of humanized mouse models with a completely functional human immune system (Brehm, Cuthbert et al. 2010; Brehm, Shultz et al. 2010). With this model, we will be able to see how the immune system is interacting with the tumor and how it can be modulated by targeting the TGFβ pathway with specific TGFβ inhibitors.

Another hypothesis that we are going to further explore is the role of Runx1 as a biomarker of response to the TβRI inhibitor. As we have described that Runx1 levels are important to mediate the TGFβ response in glioma and the mesenchymal transformation, we believe that those tumors with Runx1 would be more prone to respond to the TGFβ pathway inhibition. We are going to further validate this hypothesis *in vitro* using patient-derived neurospheres and *in vivo* with our pre-clinical xenograft model. If our hypothesis is confirmed, we could better predict and stratify those patients that can benefit from treatments with TβRI inhibitors.

CONCLUSIONS

- Glioma initiating cells (GICs) are thought to be responsible for tumor initiation and recurrence.
- GICs are characterized by expressing high levels of CD44 and *ID1*. $CD44^{high}$ population has higher tumor initiating capacity *in vivo* compared to $CD44^{low}$. This demonstrates that $CD44^{high}$ population is enriched for Glioma initiating cells.
- This population of $CD44^{high}$/Id1+ is regulated by the TGFβ pathway and can be targeted using TβRI inhibitors.
- Treatment with TβRI inhibitors leads to a differentiation in $CD44^{high}$/Id1+ cells and prevent recurrence in an *in vivo* mouse model of human GBM.
- *ID1* is crucial of GICs; thus inhibiting or decreasing its expression decreases the tumor initiating capacity of GICs.
- GICs are located in a perivascular niche, near tumor blood vessels, where there are high levels of TGFβ necessary to maintain their properties, such as self-renewal capacity or tumorigenic potential.
- Endothelial cells secrete TGFβ to the microenvironment to generate a perivascular niche for GICs.
- Treatment with TβRI inhibitor disrupts the perivascular niche of GICs and decreases the $CD44^{high}$ GIC population *in vivo*.
- $CD44^{high}$/*ID1*+ GICs are resistant to radiotherapy both *in vitro* and *in vivo*. They showed less radiation-induced apoptosis compared to $CD44^{low}$ population.
- TβRI inhibitor efficiently radiosensitizes the $CD44^{high}$ GIC population *in vitro*, demonstrating the potential benefits of combination of radiotherapy and TGFβ inhibitors in the treatment of GBM.

- One of the oncogenic effects of TGFβ is to increase self-renewal of GICs through the induction of LIF.
- Runx1 transcription factor is necessary for the TGFβ-mediated induction of LIF.
- Runx1 is necessary for GICs. Decreasing the expression of *RUNX1* causes a decrease in $CD44^{high}$/*ID1*+ population and in self-renewal capacity.
- Runx1 is also necessary to maintain an un-differentiated status. The knock-down of *RUNX1* leads to a decrease in the expression of stemness markers such as *NESTIN* and increase of astrocytic differentiation marker *GFAP*.
- Runx1 is a master regulator of the mesenchymal subclass in GBM, which is the one with worse prognosis.
- TGFβ regulates the expression of many genes from the mesenchymal gene-expression signature, such as *YKL-40*, *SERPINE1*, *RUNX1*, *LIF*, *CD44* and *ANGPTL-4*.
- The knock down of *RUNX1* decreases the expression of several of those mesenchymal genes, suggesting that Runx1 is important for TGFβ-mediated mesenchymal trans-differentiation process.
- Runx1 knock-down delays tumor formation *in vivo* suggesting an important role of Runx1 in glioma initiation.
- Tumors generated from Runx1 knock-down cells show less levels of mesenchymal markers and expression of *OLIG-2* proneural marker.
- Levels of Runx1 are higher in glioma compared to normal brain, and correlate with tumor grade.
- Runx1 is a poor prognosis factor in glioma patients. Patients with higher levels of *RUNX1* expression showed a decrease in overall survival.

RESUM DE LA TESI DOCTORAL EN CATALÀ

INTRODUCCIÓ

Glioma

El glioma es un dels tumors localitzat en el Sistema Nerviós Central (SNC). La seva forma més maligna, el Glioblastoma (GBM) de Grau IV es caracteritza per una atípia nuclear, hiperproliferació, necrosis i proliferació de les cèl·lules endotelials. El GBM és pràcticament incurable. La supervivència mitja dels pacients és de tan sols 15 mesos amb la teràpia estàndard que es basa en l'ús de quimioteràpia (temozolamida) en combinació amb radioteràpia (Stupp, Mason et al. 2005).

Les principals característiques del GBM són resumides en la Figura 1.3 (Kotliarova and Fine 2012).

- Proliferació: el GBM és un tumor amb alt grau de proliferació, en part degut a la desregulació de diferents receptors amb activitat Tirosina-Cinasa (RTKs) com és el cas del receptor del factor de creixement epidermal (EGFR). Els GBMs tenen també una desregulació en diverses vies de senyalització com ara PI3K i MAPK.
- Metabolisme: el GBM, com molts altres tumors, es caracteritza per una desregulació en el metabolisme de la glucosa, conegut com Efecte Warburg.
- Angiogenesis: els GBMs són altament angiogènics. El principal mediador d'aquest procés és el factor de creixement vascular endotelial (VEGF).
- Invasió: una de les principals característiques del GBM és que és l'elevada invasió que causa una destrucció del teixit

cerebral. La desregulació de les vies de senyalització de PI3K, MAPK i MET estan relacionades amb la invasió.

Els GBMs es poden dividir en: primaris (o *de novo*) i secundaris, que provenen de una lesió de baix grau que ha progressat a alt grau.

Les principals alteracions moleculars que caracteritzen el GBM estàn resumides en la Figura 1.6 (Parsons, Jones et al. 2008). Bàsicament trobem alteracions en les vies de senyalització de diversos RTKs (PI3K, MAPK) degudes a mutacions activadores o sobre-expressió de diversos receptors (EGFR, MET, PDGFRA i HER2) i a la deleció o mutacions inactivadores en gens que regulen aquestes vies com és el cas de *NF1* i *PTEN*. També son freqüents les alteracions en la via de p53 i la via de Retinoblastoma (Rb).

Els GBMs son molt heterogenis i es poden classificar en 4 sub-tipus (Figura 1.6) (Phillips, Kharbanda et al. 2006; Verhaak, Hoadley et al. 2010):

- **Clàssics**: es caracteritzen per una amplificació del cromosoma 7, una deleció de *CDKN2A* i del cromosoma 10 i una amplificació o mutació de *EGFR*.
- **Mesenquimals:** presenten mutacions inactivadores o pèrdua de *NF1*, *TP53* i *PTEN*, juntament amb alteracions en *MET*, *PTEN*, *CDKN2A* i *RB*. Els pacients d'aquest subgrup son els que tenen pitjor pronòstic. Es caracteritzen per l'expressió de *CD44*, *LIF*, *MET* i *YKL-40*.
- **Proneurals:** presenten mutacions en *IDH1* o *IDH2* juntament amb amplificacions en *PDGF-R* o *PDGFA* i mutacions en la via de PI3K. També presenten delecions en *TP53*, *CDKN2A* i *PTEN*. Típicament, expressen el marcador *OLIG-2*

- **Neurals:** similars als tumors de tipus clàssic però amb mutacions en *TP53* i amplificació de *EGFR*. Es caracteritzen per una elevada expressió de marcadors neuronals.

Degut a l'alta mortalitat del glioblastoma, actualment s'estan desenvolupant diversos compostos dissenyats per actuar contra aquestes vies que es troben desregulades en el glioma. Per exemple, s'han dut a terme assajos clínics per a provar compostos que inhibeixen EGFR, PDGFR, la via de PI3K o VEGF i el seu paper sobre l'angiogènesi tumoral (Tanaka, Louis et al. 2013).

Per a entendre més sobre el glioma, i testar noves teràpies, és important l'ús de models animals. Trobem dos tipus principals de models animals de glioma:

- **Models animals modificats genèticament**: es tracta de ratolins transgènics en els que s'expressa un determinat oncogen sota el control d'un promotor específic de teixit nerviós.
- **Implantació de cèl·lules tumorals:** Si les cèl·lules tumorals provenen del mateix animal o de la mateixa espècie, parlem de *allograft*, mentre que si les cèl·lules implantades són d'una espècie diferent, es tracta d'un *xenograft*. El més comú es implantar cèl·lules provinents de tumors humans en ratolins. Si aquestes s'implanten en el mateix lloc del tumor original (en aquest cas, el cervell), parlarem de un *xenograft* ortotòpic mentre que si s'implanten en un lloc diferent (sovint subcutàniament) parlarem d'un *xenograft* heterotòpic (Figura 1.10).

En aquesta tesi s'ha fet servir un *xenograft* ortotòpic de glioma inoculant cèl·lules derivades de pacients, que recapitula les característiques principals del tumor original com ara les alteracions genètiques i moleculars i la heterogeneïtat tumoral (Figura 1.11) (Anido, Saez-Borderias et al. 2010)

Cèl·lules Iniciadores de Glioma

Dins la massa tumoral podem distingir diferents tipus cel·lulars o poblacions. A part de la heterogeneïtat inter-tumoral (diferències entre pacients) també podem parlar de heterogeneïtat intra-tumoral, és a dir, en un mateix tumor trobem diferents poblacions de cèl·lules amb diferents característiques. Segons la teoria del model jeràrquic (Figura 1.12), una petita població dins del tumor té la capacitat per iniciar el creixement tumoral i generar la resta de poblacions cel·lulars (Reya, Morrison et al. 2001). Aquestes cèl·lules s'anomenen Cèl·lules Iniciadores Tumorals (o Cèl·lules Iniciadores de Glioma – GICs) i tenen algunes semblances amb les cèl·lules mare tals com la pluripotència i la capacitat d'auto renovar-se mantenint així la població de GICs. Una característica important de les GICs és la seva resistència a les teràpies convencionals que afecten al ADN, com ara la quimio i la radioteràpia. L'estudi i la caracterització d'aquesta població de cèl·lules és altament important ja que es consideren responsables de la iniciació tumoral i també de la recurrència després del tractament. S'han dissenyat alguns fàrmacs que actuen sobre les vies que es coneix estan hiperactivades en aquestes cèl·lules, com ara la via de Notch.

Aquestes cèl·lules necessiten un micro-ambient determinat amb un conjunt de factors de creixement i citoquines que els permetin mantenir

els seu estatus indiferenciat (Visvader and Lindeman 2008). S'ha descrit que aquestes cèl·lules es localitzen en nínxols determinats, com per exemple al voltant dels vasos tumorals en el cas de les GICs (Calabrese, Poppleton et al. 2007; Gilbertson and Rich 2007).

La via de senyalització de TGFβ

El factor de creixement tumoral β (TGFβ) va ser descobert l'any 1984 (Massague 1985). Pertany a una extensa família de citoquines implicades en el desenvolupament i manteniment de la homeòstasi tissular (Massague 2012). El TGFβ s'uneix al conjunt de receptors tipus I i II que tenen activitat Serina/Treonina Cinasa, i fosforilen els factors de transcripció Smads. La unió de Smad2/3 amb Smad4 envia el complex al nucli, on s'unirà a altres cofactors necessaris per a l'activació o repressió de l'expressió gènica. A part de la via clàssica, el TGFβ també pot activar altres vies com ara PI3K i MAPK (Massague and Chen 2000; Massague, Seoane et al. 2005; Ikushima and Miyazono 2010).

Típicament, el TGFβ té un efecte anti-proliferatiu bloquejant el cicle cel·lular. Però recentment s'han descobert diversos tipus tumorals en els que aquesta citoquina actua com a oncogen promovent la divisió cel·lular, la invasió i metàstasi, la angiogènesis i la supressió del sistema imune. Les funcions oncogènques del TGFβ es troben resumides en la Figura 1.16 (Blobe, Schiemann et al. 2000; Yingling, Blanchard et al. 2004; Massague 2008; Ikushima and Miyazono 2010). Un dels principals efectes oncogènics que exerceix el TGFβ és la inducció de la transició Epitelio-Mesenquimal (EMT), pas necessari per a la disseminació i metàstasi de les cèl·lules tumorals (Padua and Massague 2009; Heldin, Vanlandewijck et al. 2012). El TGFβ també confereix quimio i radioresistència a les cèl·lules tumorals. La

inhibició farmacològica de la via de TGFβ augmenta la sensibilitat de les cèl·lules tumorals a la radioteràpia, tant *in vitro* com *in vivo* (Zhang, Kleber et al. 2011; Hardee, Marciscano et al. 2012).

En el cas del glioma, el TGFβ té un clar paper com a oncogen i la hiperactivació de la via de senyalitazció correlaciona amb un pitjor pronòstic dels pacients. El TGFβ incrementa la proliferació de les cèl·lules a través de la inducció del factor de creixement PDGFB (Bruna, Darken et al. 2007) i manté la capacitat d'auto-renovació de les GICs a través de la inducció de la citoquina LIF i els factors de transcripció Sox2 i Sox4 (Ikushima, Todo et al. 2009; Penuelas, Anido et al. 2009; Seoane 2009). Altres característiques del GBM com ara la invasió, l'angiogènesi i la supressió de la resposta inmune, també estan mediades per el TGFβ (Joseph, Balasubramaniyan et al. 2013) (Figura 1.19). Això fa que s'estiguin desenvolupant diverses estratègies terapèutiques encaminades a bloquejar l'activació d'aquesta via (Figura 1.22) (Yingling, Blanchard et al. 2004; Seoane 2008). Les diferents estratègies passen per bloquejar la secreció de TGFβ mitjançant oligonucleòtids anti-sentit (AP-12009), impedir la interacció del TGFβ amb el seu receptor (anticòs anti- TGFβ GC-1008) i inhibir l'activitat enzimàtica del receptor, bloquejant així l'activació de la via de senyalització (LY2157299). Alguns d'aquests compostos s'estan provant en assajos clínics en diferents tipus tumorals.

Factors de transcripció Runx1

RUNX1 pertany a una família de factors de transcripció, també coneguts com AML (Leucèmia Mieloide Aguda, *Acute Myeloid Leukemia*) o CBF (Factors d'unió al Core, *Core Binding Factors*). Aquests gens es

caracteritzen per la presència del domini de tipus Runt, evolutivament conservat i responsable de la unió al ADN.

Aquests gens tenen moltes funcions descrites, sobretot en el desenvolupament. El factor de transcripció Runx1 té un paper clau en el desenvolupament de les cèl·lules hematopoètiques, ja que el *knock-out* del gen *RUNX1* causa una mort prematura en l'embrió degut a falta de maduració de cèl·lules sanguínies (Wang, Stacy et al. 1996). També té un paper rellevant en humans, ja que la translocació dels cromosomes t8:22 és una causa freqüent de leucèmia mieloide aguda (AML) en la que el domini d'unió al ADN (Runt) es fusiona amb el repressor transcripcional ETO1, creant un dominant negatiu de Runx1(Okuda, van Deursen et al. 1996).

Els diferents membres de la família Runx han estat implicats en diversos tipus de càncer. Runx1 s'ha estudiat àmpliament en el cas de la leucèmia, però recentment s'ha vist implicat en altres tipus de càncers, sobretot de tipus epitelial (Ito 2004; Scheitz, Lee et al. 2012).

Els factors de transcripció Runx interaccionen amb diferents vies de senyalització, i la seva funció depèn dels cofactors amb els que s'uneixen. Segons el context cel·lular, determinaran una o altra resposta transcripcional i poden actuar com a oncògens o supressors tumorals (Blyth, Cameron et al. 2005).

S'ha descrit que Runx1 es relaciona amb la via de TGFβ en diversos nivells. En primer lloc, Runx1 s'uneix físicament als factors de transcripció Smads i cooperen en la inducció de certs gens (Hanai, Chen et al. 1999; Pardali, Xie et al. 2000; Zhang and Derynck 2000). TGFβ indueix l'expressió de RUNX1 i alhora RUNX1 regula l'expressió del receptor de TGFβ potenciant l'activitat de la via (Ito and Miyazono 2003; Miyazono, Maeda et al. 2004).

RESULTATS

Donada la importància clínica de les cèl·lules Iniciadores de Glioma (GICs), vam decidir estudiar el paper de la via de TGFβ en la seva regulació, i com l'ús de inhibidors d'aquesta via pot afectar a les GICs.

La senyalització de TGFβ es important per mantenir la població de cèl·lules iniciadores de glioma (GICs)

Entre les diverses funcions oncogèniques del TGFβ, en aquesta tesi m'he centrat en l'efecte sobre la població de les GICs. Per tal d'entendre la resposta al inhibidor de TGFβ, es van analitzar els canvis en l'expressió gènica de cèl·lules derivades de pacients en resposta al tractament *in vitro* amb un inhibidor específic del Receptor tipus I de TGFβ (TβRI). Entre els diversos gens modulats per el TβRI es troben *ID1* i *ID3* (Figura 3.1), gens coneguts per el seu paper regulant el cicle cel·lular i la diferenciació de cèl·lules mare (Ruzinova and Benezra 2003). El tractament *in vivo* amb l'inhibidor TβRI també provocava una disminució dels nivells de Id1 en els tumors derivats de pacients, i una disminució de la mida tumoral (Figura 3.2). Vam comprovar que Id1 correlacionava amb el marcador de cèl·lules iniciadores CD44 (Figura 3.3) i vam demostrar que les cèl·lules amb alts nivells de CD44 ($CD44^{high}$) tenien capacitat iniciadora tumoral *in vivo* (Figura 3.4). La inhibició farmacològica de la via de TGFβ provoca una disminució de la població de GICs $CD44^{high}$/Id1+ tant *in vitro* com *in vivo* (Figura 3.5 i 3.6). Aquestes cèl·lules amb capacitat iniciadora i caracteritzades per els marcadors $CD44^{high}$/Id1+, es troben al voltant dels vasos sanguinis tumorals en alguns pacients de GBM (Figura 3.7).

Les cèl·lules endotelials secreten TGFβ creant un nínxol perivascular necessari per mantenir la població de GICs CD44^{high}/Id1

Al constatar que les GICs CD44^{high}/Id1+ es troben al voltant dels vasos sanguinis tumorals, vam pensar que era perquè en aquella zona hi havia els factors de creixement o citoquines necessaris per a mantenir les seves propietats tal com l'estat indiferenciat o la capacitat d'auto-renovar-se. Com ja hem descrit, el TGFβ té un paper rellevant en el manteniment de les propietats de les GICs, per tant, vam investigar si en aquest nínxol perivascular hi havia presència de TGFβ i si aquest era responsable del manteniment de les GICs. Primerament vam analitzar els nivells de TGFβ1 i 2 secretats per les cèl·lules endotelials i vam veure que secretaven TGFβ al medi (Figura 3.9). Aquest TGFβ secretat per les cèl·lules endotelials és capaç d'activar la via de senyalització de TGFβ, provocant la fosforilació de Smad2 i la inducció dels gens típics de resposta a TGFβ. El que és interessant és que la inducció de *ID1* és molt superior al incubar les cèl·lules amb el medi condicionat per les cèl·lules endotelials, que no pas al tractar amb TGFβ recombinant. Això ens fa pensar que hi ha alguna altra citoquina o factor de creixement que és secretat per les cèl·lules endotelials i que provoca la inducció de *ID1* (Figura 3.10). El medi condicionat per les cèl·lules endotelials també provoca un augment en la població de GICs CD44^{high} i en la capacitat d'auto-renovació de les cèl·lules tumorals derivades de pacients (Figura 3.11 i 3.12). Alhora, també provoca un increment en la capacitat tumorogènica d'aquestes cèl·lules, ja que les cèl·lules que han estat tractades amb el medi condicionat per les cèl·lules endotelials, generen tumors molt més aviat i molt més grans que les cèl·lules control. Aquest efecte és revertit amb l'inhibidor de TGFβ, demostrant així que el TGFβ és responsable d'aquest efecte pro-tumorogènic (Figura 3.13). En el nostre model de *xenograft* derivat de

cèl·lules tumorals de pacients, també observem un enriquiment perivascular de CD44 i de TGFβ. El tractament *in vivo* amb l'inhibidor de TGFβ disminueix significativament els nivells de CD44 i fa que les cèl·lules positives no es trobin al voltant dels vasos tumorals (Figura 3.14). Els nostres resultats demostren la presència de TGFβ en la zona perivascular en mostres de glioma i que les cèl·lules endotelials secreten aquest TGFβ. Això crea un nínxol perivascular on resideixen les GICs $CD44^{high}$/Id1+que requereixen dels nivells de TGFβ del medi per tal de mantenir les seves propietats tal com l'auto-renovació, la pluripotencialitat o la capacitat tumoral.

El TGFβ està implicat en el mecanisme de radio-resistència de les Cèl·lules Iniciadores de Glioma (GICs)

Una de les principals causes de fallida terapèutica en els pacients de glioma és la gran proporció de recidives després del tractament. En gran part, hom creu que és degut a la resistència de les GICs a teràpies convencionals basades en el dany a l'estructura del ADN, com ara la quimioteràpia i la radioteràpia (Bao, Wu et al. 2006; Rich 2007). Primerament vam confirmar que la població $CD44^{high}$ era resistent a la radiació γ. Vam irradiar *in vitro* diferents neuroesferes derivades de pacients i vam observar en tots els casos, un augment del percentatge de cèl·lules $CD44^{high}$ (Figura 3.16). *In vivo* també vam observar un augment de la població $CD44^{high}$/Id1+en tumors derivats de pacients després d'irradiar els ratolins a una dosi equivalent a la que es dóna en la radioteràpia en pacients de glioma (Figura 3.17).

Alguns autors postulen que el TGFβ podria ser responsable de la radioresistència de les GICs tot i que encara no està clar quin és el

mecanisme molecular implicat en aquest efecte (Zhang, Kleber et al. 2011; Hardee, Marciscano et al. 2012). El fet que l'inhibidor de TGFβ actuï sobre la població de GICs CD44^{high}/ID1+, ens va fer pensar que la combinació de la radioteràpia amb l'inhibidor de TβRI podria ser efectiva. Al irradiar *in vitro* diferents neuroesferes derivades de pacients, vam observar que el tractament amb l'inhibidor de TβRI radosensibilitzava les cèl·lules, prevenint així l'augment en la proporció de CD44^{high} així com l' inducció de ID1 (Figura 3.18). La combinació d'ambdós tractaments disminueix la proliferació i augmenta la mort cel·lular per apoptosi, sobretot tornant més sensible a la irradiació a la població de cèl·lules CD44^{high} (Figura 3.19 i 3.20). Creiem que la combinació de l'inhibidor de TβRI i la radioteràpia, al disminuir la proporció de GICs i tornar-les més sensibles a la irradiació, podria ser efectiva en prevenir les recidives en pacients de glioma.

Runx1 és un mediador de l'efecte oncogènic del TGFβ en glioma

Ja hem comentat l'efecte oncogènic que té el TGFβ en glioma, en especial regulant la població de GICs. Un dels principals mediadors d'aquest efecte oncogènic del TGFβ en glioma és la inducció de la citoquina LIF, que regula la capacitat d'auto-renovació de les GICs (Penuelas, Anido et al. 2009). Per tal d'entendre millor aquest efecte dual del TGFβ en càncer i com regula a nivell molecular la inducció de LIF, vam estudiar el promotor del gen *LIF* i com s'indueix per TGFβ. Resultats previs identifiquen la regió del promotor responsable de la inducció de *LIF* per TGFβ i el lloc d'unió dels factors de transcripció Smads. Però sabem que els Smads tenen una baixa afinitat per el ADN i que requereixen de cofactors, per tant vam identificar un lloc d'unió a Runx1 en una regió propera al lloc d'unió de Smads (Figura 3.22). La mutagènesi dirigida d'aquest lloc d'unió a Runx1 impedeix la inducció de

LIF per TGFβ, demostrant així la importància de Runx1 com a mediador de la inducció de *LIF* per TGFβ (Figura 3.23). Experiments de immunoprecipitació de cromatina (ChIP) demostren que Runx1 s'uneix al promotor de *LIF* (Figura 3.24). Per tal d'estudiar el paper de Runx1 com a mediador de l'efecte oncogènic del TGFβ en glioma, vam disminuïr la seva expressió mitjançant interferència de ARN (siRNA) i una forquilla d'interferència (shRNA). Vam utilitzar aquesta estratègia tant en línies cel·lulars de glioma (U373-MG i U87-MG) com en neuroesferes derivades de pacients (GBM2, GBM3 i GBM7). En tots els casos, vam observar una disminució en els nivells de LIF al disminuir Runx1, demostrant així la importància de Runx1 com a mediador de la inducció de LIF (Figura 3.25 i 3.26). Per altra banda, vam sobre-expressar *RUNX1* en cèl·lules de glioma i en neuroesferes derivades de pacients i vam observar que els nivells de LIF augmentaven i també la inducció per TGFβ (Figura 3.27). Al analitzar l'expressió de *LIF* i *RUNX1* en 374 mostres de glioma, vam observar una correlació estadísticament significativa ($p<0.0001$) (Figura 3.28). Degut a que LIF té un paper rellevant sobre la població de GICs, vam pensar que potser Runx1 també era necessari per al manteniment d'aquestes. Vam observar que al disminuir els nivells de Runx1, també disminuïa la població de GICs $CD44^{high}$ (Figura 3.29). Al sobre-expressar *RUNX1* vam observar un augment de la població $CD44^{high}$, confirmant així el paper de Runx1 mantenint la població de GICs $CD44^{high}$ (Figura 3.30). Runx1 també és necessari per l'auto-renovació de les GICs, ja que al disminuir els nivells de Runx1, es perd la capacitat d'auto-renovació de les neuroesferes, i al sobre-expressar *RUNX1* augmenta la capacitat d'auto-renovació de les neuroesferes derivades de pacients (Figura 3.31 i 3.32). LIF és necessari per mantenir les GICs en un estat indiferenciat, caracteritzat per l'expressió de certs marcadors típics de cèl·lules mare com ara *NESTIN*, *SOX2* o *MUSASHI*-

1. La disminució dels nivells de Runx1 provoca una disminució en l'expressió de *NESTIN* i *SOX2* i mentre que l'expressió de *GFAP*, marcador de diferenciació d'astròcits, augmenta, suggerint que la falta de Runx1 provocaria una diferenciació de les GICs cap a un fenotip d'astròcits (Figura 3.33 i 3.34). Altres marcadors de diferenciació com ara Tuj1 (marcador de diferenciació neuronal) o O4 (marcador de diferenciació oligodendrocític) no es veuen afectats per la modulació de Runx1.

S'ha descrit que Runx1 és un dels 6 factors de transcripció necessaris per al fenotip mesenquimal dels glioblastomes (Carro, Lim et al. 2010). Molts dels gens que es troben sobre-expressats en aquest subtipus tumoral són regulats per l'activitat TGFβ, cosa que ens porta a pensar que Runx1 podria mediar, no només la inducció de LIF per TGFβ, sino també la inducció d'altres gens típics del fenotip mesenquimal. La disminució dels nivells de Runx1 en cèl·lules derivades de pacient, provoca una disminució en l'expressió de diversos marcadors mesenquimals i de la seva inducció per TGFβ. Per exemple *LIF, SERPINE-1, AGPTL-4 i YKL-40* es veuen afectats per la disminució de Runx1 (Figura 3.36). En el cas de les neuroesferes derivades d'un tumor amb un perfil d'expressió mesenquimal, al disminuïr els nivells de Runx1 vam observar un augment en els marcadors pro-neurals *OLIG-2* i *BCAN* (Figura 3.36 B). De manera similar, al sobre-expressar *RUNX1* en neuroesferes derivades d'un tumor de tipus proneural, vam observar un augment en els marcadors mesenquimals *LIF*, *SERPINE-1* i *AGPTL-4* i una disminució en els marcadors proneurals *OLIG-2* i *BCAN* (Figura 3.37).

Per tal d'estudiar el rol del factor de transcripció Runx1 en el glioma *in vivo* vam inocular en el cervell de ratolins immunosuprimits, neuroesferes derivades de pacients amb una disminució de Runx1. Aquestes

neuroesferes eren menys eficients a l'hora de generar tumors comparades amb les neuroeferes control. En el moment en que tots els animals on s'havien inoculat neuroesferes control havien desenvolupat tumors importants, cap dels ratolins que havien estat inoculats amb cèl·lules on s'havien disminuït els nivells de Runx1 havia generat tumors, i els ratolins d'aquest grup tenien una supervivència significativament major (Figura 3.38). Al cap del temps, però, les cèl·lules amb baixos nivells de Runx1 acabaven generant tumors, suggerint que el paper més important de Runx1 seria durant la fase de iniciació tumoral. En un altre experiment independent, vam inocular cèl·lules que expressen de forma constitutiva Luciferasa, de manera que podem quantificar el tumor per bioluminescència. Les cèl·lules amb una disminució de Runx1 van generar tumors significativament més petits que les cèl·lules control (Figura 3.38). Al fer l'experiment contrari, i inocular cèl·lules que sobre-expressen *RUNX1*, vam observar l'efecte oposat, és a dir, les cèl·lules amb alts nivells de Runx1 generaven tumors significativament més grans que les cèl·lules control (Figura 3.41), demostrant així el paper de Runx1 en la iniciació i progressió del glioma *in vivo.* Al estudiar les característiques dels tumors formats, vam observar que els nivells de LIF, Nestin, YKL-40 en els tumors generats per les cèl·lules on haviem disminuït Runx1, eren menors que en els tumors control. Per altra banda, en alguns casos, vam observar un augment de Olig-2, suggerint que els tumors perdien les característiques mesenquimals i eren de tipus proneural al disminuïr Runx1 (Figura 3.39 i 3.40).

Al analitzar els nivells de Runx1 en mostres de glioma, vam observar que Runx1 es troba més elevat en teixit tumoral en comparació amb teixit sa i que dins dels diferents tipus de glioma, els nivells són més elevats en els tipus més maligne, glioblastoma (Figura 3.42). L'anàlisi de dades clíniques

mostra que els pacients on *RUNX1* està sobre-expressat, tenen pitjor pronòstic, implicant així Runx1 com a marcador de mal pronòstic en glioma (Figura 3.43).

DISCUSSIÓ

El glioblastoma és un dels tipus de tumors amb pitjor pronòstic, amb una supervivència mitja de només 15 mesos. Tot i que cada dia coneixem millor les seves característiques moleculars, aquests avenços no es tradueixen en millores en el tractament dels pacients. És per això que cal trobar noves aproximacions terapèutiques.

Durant aquesta tesi, he centrat els meus estudis en la via de senyalització de TGFβ i en el seu paper oncogènic en glioma. El paper del TGFβ com a oncogen ha estat molt estudiat, sobretot en carcinomes en els quals indueix la proliferació, la transició epitel·lio-mesenquimal, la metàstasi, l'angiogènesi i la supressió del sistema immune (Massague 2008). Sobretot m'he centrat en el rol oncogènic del TGFβ sobre les Cèl·lules Iniciadores de Glioma (GICs). Aquestes cèl·lules són pluripotents, tenen capacitat d'auto-renovar-se i poden diferenciar-se en els diferents tipus cel·lulars que formen el tumor. L' interès que susciten aquestes cèl·lules ve donat pel fet que són resistents a les teràpies convencionals que danyen l'estructura del ADN, com ara la quimio i la radioteràpia. Aquestes cèl·lules són capaces de re-iniciar el tumor després del tractament causant una recurrència. És per això que estem interessats en estudiar la regulació d'aquestes cèl·lules per tal de trobar noves dianes terapèutiques que puguin atacar aquesta població.

Per tal de poder traslladar els descobriments fets al laboratori a la pràctica clínica, fem servir un model de recerca traslacional. En aquest sentit, la col·laboració amb l'hospital ens permet obtenir mostres de tumors de pacients, dels quals derivem cèl·lules i també obtenim ADN i ARN per tal d'analitzar les mutacions i el perfil d'expressió. També inoculem de manera sistemàtica aquestes cèl·lules derivades de pacients en ratolins immunosuprimits, per tal de generar tumors que recapitulen les característiques del tumor del pacient: histologia, expressió de biomarcadors, heterogeneïtat i alteracions gèniques. Aquest model ens permet provar diferents fàrmacs que s'estan començant a donar en la clínica i entendre els mecanismes de resposta i resistència als tractaments.

Sabem que la via de TGFβ és important per al manteniment de les GICs, així que vam estudiar la resposta del inhibidor de TGFβ (LY 2019761). Al tractar cèl·lules derivades de pacients amb aquest inhibidor, vam veure que disminuïa l'expressió de *ID1* i *ID3,* suggerint que podien ser marcadors d'aquesta població de GICs. Vam estudiar com correlacionava amb diversos marcadors descrits per les GICs, com son CD44 (descrit en càncer de mama), CD133, SEEA-1 o ALDH1, i vam observar que l'expressió de *ID1* correlacionava amb alts nivells de CD44 ($CD44^{high}$), però no amb altres marcadors de cèl·lules mare tumorals. Per tal de demostrar que aquesta població de cèl·lules $CD44^{high}$/Id1+ tenen realment capacitat iniciadora tumoral, vam realitzar assajos de dilucions límit *in vivo* inoculant quantitats decreixents de cèl·lules en ratolins immunosuprimits. Mentre que amb tan sols 100 cèl·lules $CD44^{high}$ observàvem algun tumor, la mateixa quantitat o quantitats superiors de $CD44^{low}$ no generaven tumors *in vivo*. El fet de descriure aquests nous marcadors de GICs, en especial $CD44^{high}$ que és un receptor de membrana, ens permet identificar aquesta població de GICs i per tant estudiar-la i provar noves teràpies dirigides contra les GICs.

Una troballa important és el fet que les cèl·lules endotelials secreten TGFβ i que aquest és necessari per mantenir les GICs i les seves propietats. Això posa de manifest la importància que té el micro-ambient tumoral. Vam observar que les GICs tendien a localitzar-se en la perifèria dels vasos sanguinis tumorals en alguns pacients de GBM. Això ens va portar a pensar que potser les cèl·lules endotelials secretaven algun factor de creixement o molècula que podria ser important per a les GICs. Ens vam centrar en el paper del TGFβ, que és secretat al medi per les cèl·lules endotelials i que és capaç d'activar la senyalització en neuroesferes derivades de pacients, a la vegada que manté la població de GICs $CD44^{high}$/Id1+ i la seva capacitat oncogènica. El que és interessant és que la inducció de *ID1* amb el medi condicionat per les cèl·lules endotelials és molt superior a la inducció per TGFβ, suggerint que hi ha alguna altra citoquina o factor de creixement que col·labora amb el TGFβ provocant aquest augment de *ID1*. Aquesta col·laboració serà estudiada en futurs experiments en el nostre grup.

Per tal de desenvolupar teràpies que actuïn contra les GICs i per tant disminuïr la probabilitat de recidives, cal l'estudi de les vies de senyalització que són importants. En aquest cas, sabent que TGFβ regula aquestes GICs $CD44^{high}$/Id1+, pensem que la l'ús de inhibidors de TGFβ combinats amb teràpies convencionals com la radioteràpia, pot millorar el pronòstic dels pacients. En aquesta línia, s'estan realitzant diversos assajos clínics amb compostos que inhibeixen la senyalització de TGFβ, com són ARN-antisentit, anticossos que impedeixen de la citoquina al receptor i molècules que impedeixen l'activació del receptor (Yingling, Blanchard et al. 2004; Akhurst and Hata 2012). Alguns d'aquests fàrmacs estan en assajos clínics en combinació amb quimio i radioteràpia. Els nostres resultats suggereixen que la combinació de teràpies anti- TGFβ amb teràpies que danyen el ADN, com ara la radioteràpia, poden ser efectives

en disminuir la població de GICs i per tant en prevenir la resistència i les recidives.

En el transcurs d'aquest treball hem identificat el factor de transcripció Runx1 com un important mediador de l'efecte oncogènic del TGFβ. Runx1 es necessari per la inducció de LIF per TGFβ i per tant per al manteniment de la capacitat d'auto-renovació de les GICs i per a mantenir-les en un estat indiferenciat. Altrament, també és necessari per la inducció de gens de la classe mesenquimal, molts dels quals són regulats per TGFβ (*LIF*, *SERPINE-1*, *AGPTL-4*, *YKL-40*). Això ens suggereix que potser TGFβ és en part responsable de la transdiferenciació del subtipus de glioma mesenquimal, i que Runx1 seria un important mediador d'aquest procés. El més interessant és que aquest subtipus és el que té pitjor pronòstic, corroborant així el paper del TGFβ com a factor de mal pronòstic. Runx1 també es un factor de mal pronòstic en pacients de glioma, aquells pacients amb nivells més elevats, tenen menys supervivència. També hem demostrat que Runx1 és necessari per la iniciació del glioma en el nostre model *in vivo*. La relació de TGFβ amb els factors de transcripció de la família de Runx ja es coneixia des de fa anys, el que nosaltres descrivim per primer cop és com aquesta relació és important en glioma i com Runx1 podria ser un dels principals mediadors de l'efecte oncogènic en glioma. La importància de Runx1 com a mediador de l'efecte oncogènic del TGFβ, va més enllà de la inducció de LIF, ja que també actua modulant l'expressió de diversos gens de la signatura mesenquimal. A més, per primer cop, descrivim la importància de Runx1 en el glioma. En una futura línia de recerca, investigarem si Runx1 es podria considerar, a més d'un mediador de la resposta al TGFβ, un mediador de resposta al inhibidor de TGFβ. Això ens permetria predir si un pacient o un altre respondrà o no al tractament amb inhibidor de TGFβ i per tant dissenyar teràpies millors.

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