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DEVELOPMENT OF LENTIVIRAL VECTORS TO STUDY THE INFLUENCE OF ANGIOGENIC MOLECULES ON GLIOMA GROWTH

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Résumé

Les glioblastomes sont des tumeurs du système nerveux central hautement létaux. Ils se caractérisent par leur grande infiltration dans les tissus avoisinants. Ils modifient les vaisseaux sanguins préexistants et ils migrent d'une façon perivasculaire. Cette cooption vasculaire est un processus entraînant l'expression d'Angiopoietine-2 (Angpt2) par des cellules endothéliales et sa liaison au récepteur Tie2. Le **premier objectif** de cette étude était d'examiner le potentiel thérapeutique de deux protéines qui pourraient interférer avec Angpt2, à savoir Angpt3 et la partie soluble extracellulaire du récepteur Tie2 (sTie2). Le **deuxième objectif** était de développer des vecteurs lentiviraux capables d'exprimer ces protéines, tout en offrant la possibilité d'identifier et détruire les cellules génétiquement modifiées. À cette fin, nous avons construit un vecteur contenant une cassette bicistronique qui exprime le marqueur amélioré de la protéine fluorescente verte (EGFP) fusionnée au gène suicide provenant du virus herpès simplex de type I-thymidine kinase (HSVtk). Les cellules du gliome GL261 transduites avec ce vecteur pourraient être suivies et tuées sur demande par l'administration de la prodrogue ganciclovir, soit *in vitro*, soit après l'implantation dans le cerveau des souris.

Malgré l'expression des hauts niveaux d'Angpt3 et de sTie2 obtenus avec ce vecteur, nous avons observé qu'Angpt3 augmente la déstabilisation capillaire et la croissance de gliomes, alors que sTie2 n'exerce aucun effet. Globalement, cette étude a permis de comprendre l'importance de la voie de signalisation de Tie2 dans le développement des gliomes et le rôle d'Angpt3, mais suggère que ni cette molécule ni sTie2 soient des agents efficaces contre les gliomes malins. Cette étude fournit également le prototype d'un vecteur lentiviral pour la thérapie génique plus sécuritaire.

Abstract

Glioblastomas are highly lethal tumors of the central nervous system characterized by large spread into the surrounding tissues. They modify and migrate along pre-existing blood vessels. This vascular cooption is a process involving the release of angiopoietin-2 (Angpt2) from endothelial cells and binding to the Tie2 receptor. The **first goal** of this study was to examine the therapeutic potential of two proteins that could interfere with Angpt2, namely Angpt3 and the soluble extracellular domain of Tie2 (sTie2). The **second goal** was to develop a lentiviral vector capable of delivering such proteins while offering the possibility to identify and destroy the genetically modified cells. To this end, we designed a bicistronic construct expressing the marker enhanced green fluorescent protein (EGFP) fused to the suicide gene herpes simplex virus 1-thymidine kinase (HSVtk). GL261 glioma cells transduced with this vector could be tracked and killed on command by the administration of the prodrug ganciclovir, either *in vitro* or after implantation into mouse brains.

High levels of Angpt3 or sTie2 could be achieved with this vector; however, Angpt3 increased capillary destabilization and glioma growth, whereas sTie2 exerted no effect. Overall, this study helps to understand the importance of the Tie2 signaling pathway in glioma development and the role of Angpt3, but suggests that neither this molecule nor sTie2 are effective agents against malignant gliomas. This study also provides a lentiviral vector design for safer gene therapy.

Resumen

Los glioblastomas son tumores del sistema nervioso central altamente letales caracterizados por su gran diseminación a tejidos vecinos de la masa tumoral. Estos tumores modifican y migran sobre vasos sanguíneos preexistentes. Esta co-opción perivascular es un proceso que involucra la expresión de Angiopoietina 2 (Angpt2) en células endoteliales y su unión al receptor Tie2. El **primer objetivo** de este estudio fue examinar el potencial terapéutico de dos proteínas capaces de interferir con Angpt2, las cuales fueron Angpt3 y el dominio soluble extracelular de Tie2 (sTie2). El **segundo objetivo** fue desarrollar un vector lentiviral capaz de expresar éstas proteínas y, al mismo tiempo, permitir la identificación y destrucción de las células genéticamente modificadas. Para esto, se construyó un cassette bicistrónico conteniendo el marcador mejorado de la proteína verde fluorescente (EGFP) fusionado al gene suicida proveniente del virus de herpes simples l-timidina kinasa (HSVtk). Células de glioma GL261 transducidas con el vector desarrollado en este estudio, fueron identificadas y destruidas de manera específica posterior a la administración de la prodroga ganciclovir, tanto *in vitro* como después de su implantación en el cerebro de ratones.

Altas concentraciones de Angpt3 o sTie2 fueron alcanzadas con este vector; sin embargo, Angpt3 aumentó la desestabilización capilar y el crecimiento del glioma, mientras que sTie2 no ejerció ningún efecto notable. A manera general, este estudio ayuda a comprender la importancia de la vía de señalizaciones Tie2 en el desarrollo del glioma y el rol de Angpt3, pero sugiere que ni esta molécula ni sTie2 son agentes eficientes en la lucha contra los gliomas malignos. Este estudio también provee el diseño de un vector lentiviral con miras a una terapia génica mucho más segura.

Avant-propos

J'aimerais, tout d'abord, remercier mon directeur de thèse le Dr Luc Vallières de m'avoir accueillie dans son laboratoire et de m'avoir montré un exemple à suivre quant à l'intégrité professionnelle ainsi que l'ouverture et la dévotion pour la recherche biomédicale. Merci, Dr Vallières, pour votre support et confiance.

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Je profite pour bien souligner mes contributions aux expériences présentes sur cette thèse. Comme noté sur le cahier du laboratoire (I et II), j'ai participé aux mis au point de la production et quantification des vecteurs lentiviraux, j'ai fait les expériences *in vitro* et *in vivo* pour déterminer l'effet cytotoxique dû prodrogue ganciclovir en différents concentrations et plusieurs jours; je fus les premiers expériences in vitro pour les cellules transduites Angpt3 et sTie2 pour finir avec des quantifications de ces tumeurs *in vivo* chez les souris C57BL/6. Comme travaux additionnels, j'ai appris la technique de hybridation *in situ* pour la molécule hAngptL4, je fus la caractérisation de 19 lignes cellulaires de gliomes humaines chez des souris SCID et j'ai collaboré à montrer l'effet sur la survie *in vivo* de la modification de l'expression des différents isoformes de hAngptL4.

Finalement, j'exprime mes remerciements à Québec. J'ai obtenu, j'ai appris et j'ai gagné beaucoup plus de gratifications que je suis venue chercher : j'ai obtenu mon diplôme avec tous les expériences que celui-ci implique, j'ai appris à cuisiner avec la liberté de déguster et mélanger tous les saveurs du monde et j'ai gagné l'amitié de personnes inoubliables. Alors, merci Québec, merci la vie.

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To all people that love me and trust me.

Especially to my sister Citlalli, who oriented my life with her short life.

To all glioma survivors [1, p56]

"I eventually came to the realization that the whole process was not just about avoiding pain and making my tumor go away, but was about enjoying and savoring life. When I started living my life that way, amazing things happened."
Meningioma survivor

''Hey, wake up, you are alive.''

Subject list

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Acronym list

5-FC	5-Fluorocytosine
5-FU	5-Fluorouracile
Δp53	Mutant protein 53
AA	Astrocytic astrocytoma
AdV	Adenoviral vector
AKT	Alpha serine/threonine-protein kinase
amp	Amplification
Angpt	Angiopoietin
Angptl	Angiopoietin-like
ANOVA	Analysis of variance
ARF	ADP-ribosylation factor 1 (tumor supressor p14)
BBB	Brain blood barrier
Bcl2	B-cell leukemia/lymphoma 2
BrdU	Bromodeoxyuridine
CBTRUS	Central brain tumor registry of the United States
CC	Cerebral cortex
CCND2	Cyclin D2
CP	Caudoputamen
CPP	Choroid plexus papilloma
CD	Cytosine deaminase
CDK4	Cyclin-dependent kinase
CDKN2	Cyclic AMP (adenosine monophosphate)-dependent kinase number 2
cDNA	Complementary DNA
Chr	Chromosome
CMV	Citomegalovirus
CNS	Central nervous system
cPPT	Central polypurine tract
CSC	Cancer stem cell
Ctrl	Control
DCC	Deleted in colorectal cancer
DNA	Deoxyribonucleic acid
dTTP	2'-deoxythymidine 5'-triphosphate
EC	External capsule
ECM	Extracelullar matrix
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
ERK	Extracellular receptor kinase
ERBB2	Erythroblastic leukemia viral oncogene homolog 2
et al	*et alia* (and others)
F	Female
F-AraAMP	9-beta-D-arabinofuranosyl-2-fluoroadenine monophosphate
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
F-dAdo	2'-deoxy-2'-fluoroadenosine
FGF	Fibroblast growth factor
FIAF	Fasting-induced adipose factor
FOX0	Forkhead box 01
GalC	Galactocerebrosidase
GBM	Glioblastoma
GCV	Ganciclovir
GFP	Green fluorescent protein
GFAP	Glial fibrillary acidic protein
HATs	Histone acetyltransferases
HCl	Acid chloride
HIF	Hypoxia inducible factor
HMGB	High mobility group box
HSV*tk*	Herpes simplex virus thymidine kinase
IL4	Interleukine-4
INK4	Inhibitor of cyclin-dependent kinase 4 (p16)
IRES	Internal ribosomal entry site
Km	Michaelis constant
LCMV-GP	Lymphocytic choriomeningitis virus-glycoproteins
LGA	Low grade astrocytoma
LOH	Loss of heterozygosity
LTR	Long terminal repeat
LV	Lentiviral
M	Male
MAX	Myc associated factor X
MBP	Myelin basic protein

MCS	Multicloning site
MDM2	Murine double minute 2
MeP-dR	6-Methylpurine-2-deoxyriboside
MET	Mesenchymal-epithelial transition factor (protooncogene tyrosine kinase (hepatocyte growth factor receptor))
MK801	Dizocilpine, antagonist of the NMDA receptor
MYC	Myelocytomatosis viral oncogene homolog (transcription factor binding E-boxes and recruiting HATs)
MRI	Magnetic resonance imaging
mRNA	messenger RNA
Mut	Mutated
N/C	Not communicated
NF1	Neurofibromatosis type 1
NF2	Neurofibromatosis type 2
NFAT	Nuclear factor of activated T cells
NIH-3T3	Mouse embryonic fibroblast cell line
NL	Nuclear localization signal
NP	Nucleoside phosphorylase
NR	No record
NURR	Nuclear receptor-related factor 1
Olig2	Oligodendrocyte lineage transcription factor 2
Overexp	Overexpressed
PA	Pilocytic astrocytoma
PAK	P21-activated kinase
PCR	Polymerase chain reaction
PDGFR	Platelet derived growth factor receptor
PEDF	Pigment epithelium-derived factor
PI3K	Phosphoinositide-3-kinase
PNP	Purine nucleoside phosphorylase
PPP	Triphosphate
Psi	Packaging signal
PTEN	Phosphatase and tensin homolog of chromosome TEN
+	Positives
RAS	Rat sarcoma
RB	Retinoblastoma
RTK	Receptor tyrosine kinase
Rig	Regulated in glioma
RT-PCR	Real time PCR
SCID	Severe combined immunodeficiency disease
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEER	Surveillance epidemiology and end results
SFM	Serum-free medium
SIN	Self –inactivating lentiviral vector
SP	Signal sequence in the amino terminal end of the gene sequence
TBVs	Tumor blood vessels
TG	Triglyceride
TIE	Tyrosine kinase with Ig and EGF homology domains
TK	Thymidine kinase
Tn C	Tenascin C
TNF	Tumor necrosis factor
TP53	Tumor protein p53
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
HIV	Human immunodeficiency virus
vp	Viral particles
WHO	World health organization
WPRE	Woodchuck hepatitis post-transcriptional regulatory element

Chapter 1: Brain tumors

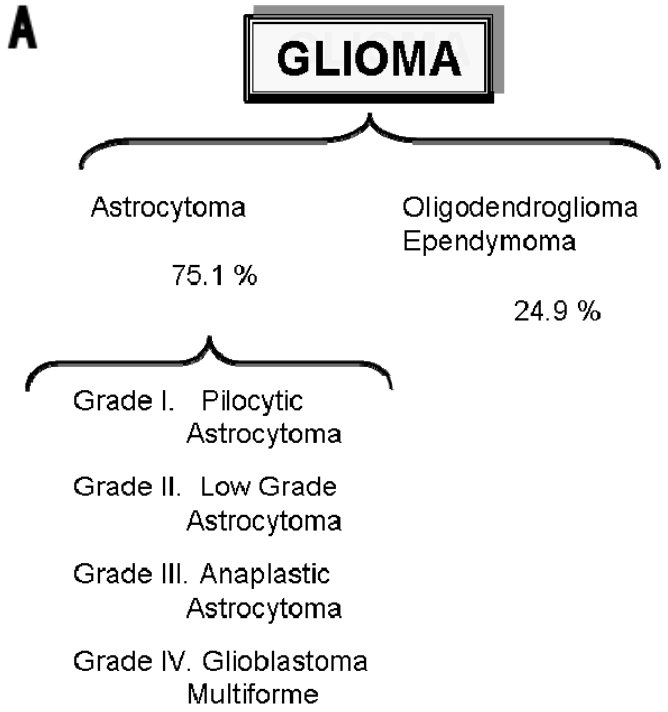
I Introduction to glioblastomas (GBMs)

I.1 Glioma biology and classification

The term glioma was introduced in 1860 by the German pathologist Rudolf Virchow to identify neuroepithelial tumors of the central nervous system (CNS). Gliomas account for 40% of all primary brain / CNS tumors and for 78% of malignant brain tumors, while astrocytomas (including GBMs) account for 75% of all gliomas (Figure 1A). So far, more than 120 brain tumors are classified according to the cell type of origin: astrocytes, oligodendrocytes, ependymal or neuronal cells. Almost 90% of these tumors are located inside the brain and only 10% in the spinal cord (Annex I, WHO Classification of brain tumors 2007 and Topography of brain tumors by grade of malignancy) [1-3].

Gliomagenesis involves one cancer stem cell (CSC) usually recognized with the molecular markers GFAP, Olig2, bTubulinIII and CD133 [4]. The accumulation of lesions in genes like NF1, NF2, TP53, EGFR and PTEN in oligodendrocytes and astrocytes produces gliomas of four possible grades of malignancy. Grade I astrocytomas are biologically benign and can be surgically cured if deemly resected at the time of diagnosis; grade II are low-grade malignancies that may follow long clinical courses and lead to death within 5–10 years; grade III are malignant and lethal within 2–5 years; grade IV are highly malignant, usually recalcitrant to chemotherapy, and lethal within 9–15 months. The high infiltration profile of each grade is demonstrated in the magnetic resonance images in figure 1B [5, 6] (for a more detailed molecular biology of survival, refer to Table 2).

It is important to note that each grade of malignancy entails histopathological characteristics and molecular markers. Also, each of them is able to develop to the next grade of malignancy over time, reaching the malignant secondary GBM. To date, *de novo* GBM or primary GBM appears like the result of genetic aberrations without previous grade of malignancy (Figure 1B) [7-9].



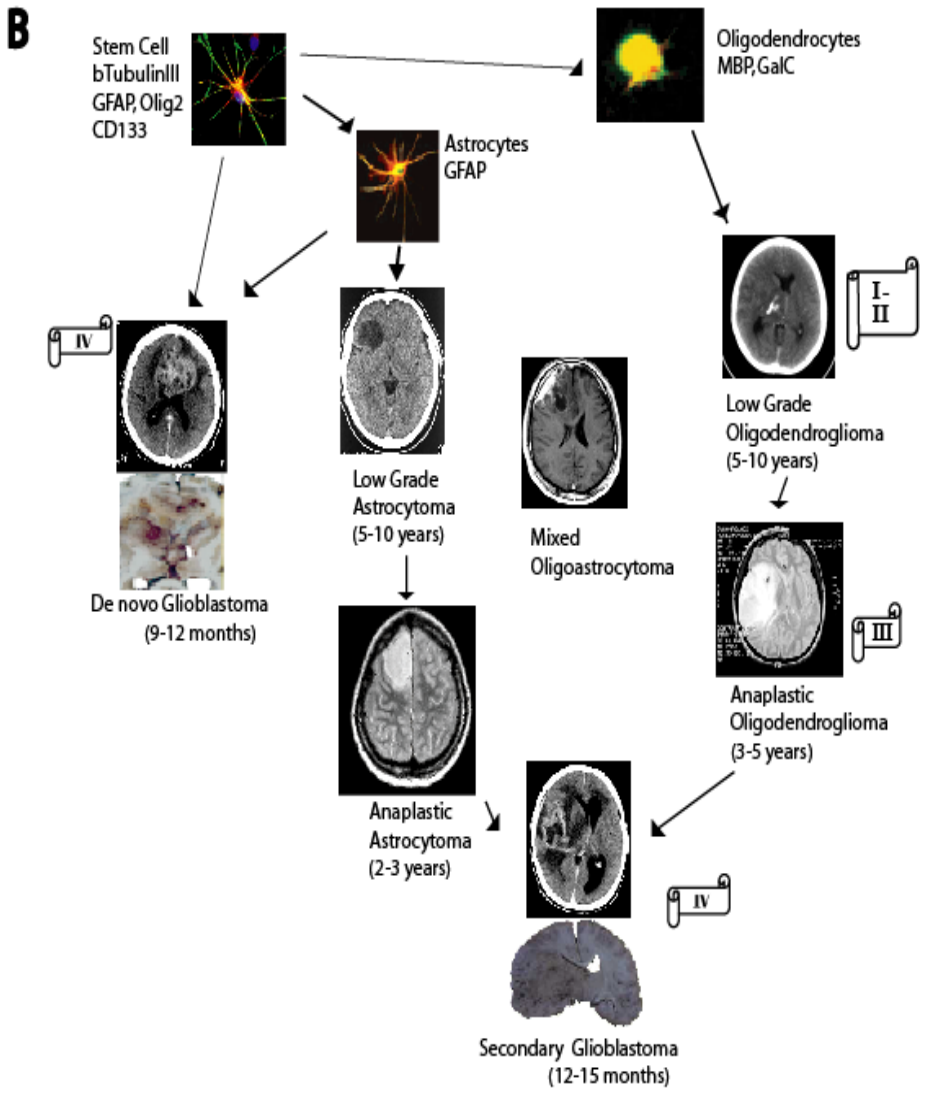


Figure 1 Glioma classification and gliomagenesis. A) 75,1% of gliomas are astrocytomas of four grades and 24,9% are composed of oligodendrogliomas, ependymomas and mixed gliomas. B) Primary and secondary GBMs evolve differently from cancer stem cells. The degree of invasion corresponds to the degree of malignancy and survival time decreases accordingly [10].

I.2 Glioblastomas

The incidence rate for all primary brain tumors by age-specific statistic is bimodal, with a peak incidence at 1-4 years-old and 70-84 years-old populations (Figure 2A). In fact, brain tumors are considered the most deadly solid neoplasm in children and ranges third in adults [1, SEER Registries 1992-2006].

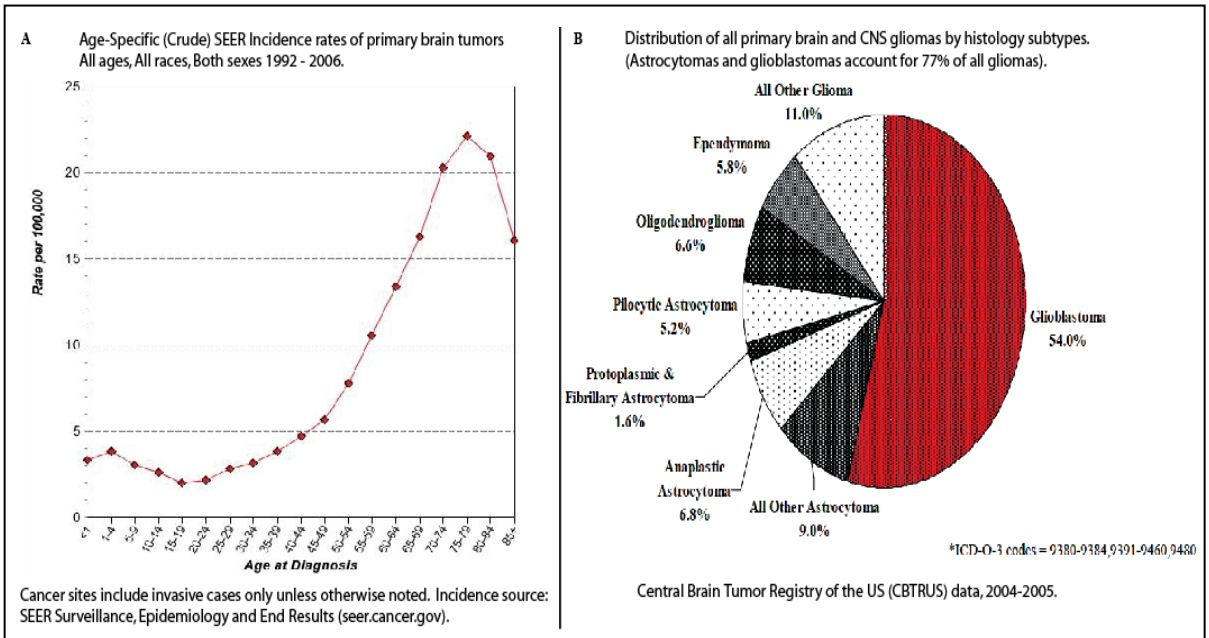


Figure 2 Incidence rates and distribution by histology of GBMs. A) Primary brain tumor incidence rates at all age groups in the last 17 years. B) GBM distribution by histology subtypes. 54% of gliomas are GBMs.

Glioblastomas (astrocytoma of grade IV) are the most common primary brain tumors, representing 54% of all gliomas, followed by others astrocytomas (22,6%), oligodendrogliomas (6,6%), ependymomas (5,8%) and other gliomas (11%) (Figure 2B). Glioblastomas have a high invasion profile characterized with palisading cells, increased cellularity, morphological atypia and nuclear anaplasia (which is the basis of the designation "multiforme"), high mitotic activity, increased microvascular density and necrotic core areas. Almost 60% of them are located in the white matter of the fronto-parieto-temporal lobes growing toward the cortex (Figure 3A) [11, Annex I, Topography of gliomas].

Table 1 Incidence rates of malignant gliomas in North America and the world.

REGION	Incidence (by 100 000 people-years)	Notes
Worldwide (1993-1997)	6,8	Low grade gliomas were 4,2 for the same period
Mexico (1995-2001)	7,8	Low grade gliomas were 4,7 for the same period
USA (2002-2006)	7,6	Incidence for women was 5,4 for the same period
Canada (2008)	7	Benign cases are not taken in the registries (30% of cases are missing)
Québec (2008)	9	Incidence for women was 6 for the same period

The order followed corresponds to the time period in which the statistical data was obtained. [12, Canadian Cancer Society Webpage, Brain Tumour Canada Webpage and seer.cancer.gov]

In general, GBM incidence is around 7/100 000 people per year, but this number is increasing due to better diagnostic techniques and, paradoxically, to the longer survival of patients suffering from other cancers that generate brain metastases. Furthermore, 75% of people afflicted by GBM die in less than 24 months, and even after surgical resection of the tumor, there is the probability to develop another glioma near the original niche due to the high invasive features of this tumor [3, 13, 14].

I.2.1 Glioblastoma classification

In order to better understand GBM biology, they are classified according to the main cell type (astrocytic, oligodendrocytic or ependymal) from which the glioma evolved. Primary GBMs develop clinically *de novo* and secondary GBMs evolve from low-grade gliomas (WHO grade II) and anaplastic astrocytomas–oligodendrogliomas (WHO grade III). Recent studies have shown a relationship between histopathology and molecular or genomic rearrangements, overexpression, mutation and knockdown of key genes. This molecular profile is used today to describe preferential brain locations [15, 16], chemotherapy response [17], survival clusters [18], etc. (Figure 3A).

The key genes modified in GBMs have been clustered to show that the three cell functions and critical signaling pathways affected are: the regulation of growth factor signals by EGFR/PDGF/MET amplification and PTEN/NF1/FOXO mutation (RTK/RAS/PI3K pathway), the response to DNA damage and cell death (apoptosis and senescence) by direct mutation of the p53 pathway and regulation of cell division affecting the CDK/cyclin/CDK inhibitor/RB pathway [10] (Figure 3B).

Physically, the stages required for GBM cells to grow in the brain are: Stage I implantation (the first tumor cells appear dispersed within the neuropil); stage II perivascular organization (tumor cells are concentrated around nutrient-rich native blood vessels); stage III proliferation of the tumor cells around the still viable blood vessels; stage IV apoptosis and involution where apoptosis in vascular cells occurs, and degeneration of the blood vessels becomes evident. The involution of the host vessels is likely to lead to hypoxia, which in turn will induce VEGF release, leading to angiogenesis. Lastly, the stage V angiogenesis involves neovascularization, new blood vessels grow toward and vascularise the necrosing tumor core providing a new source of nutrition [19] (Figure 3C).

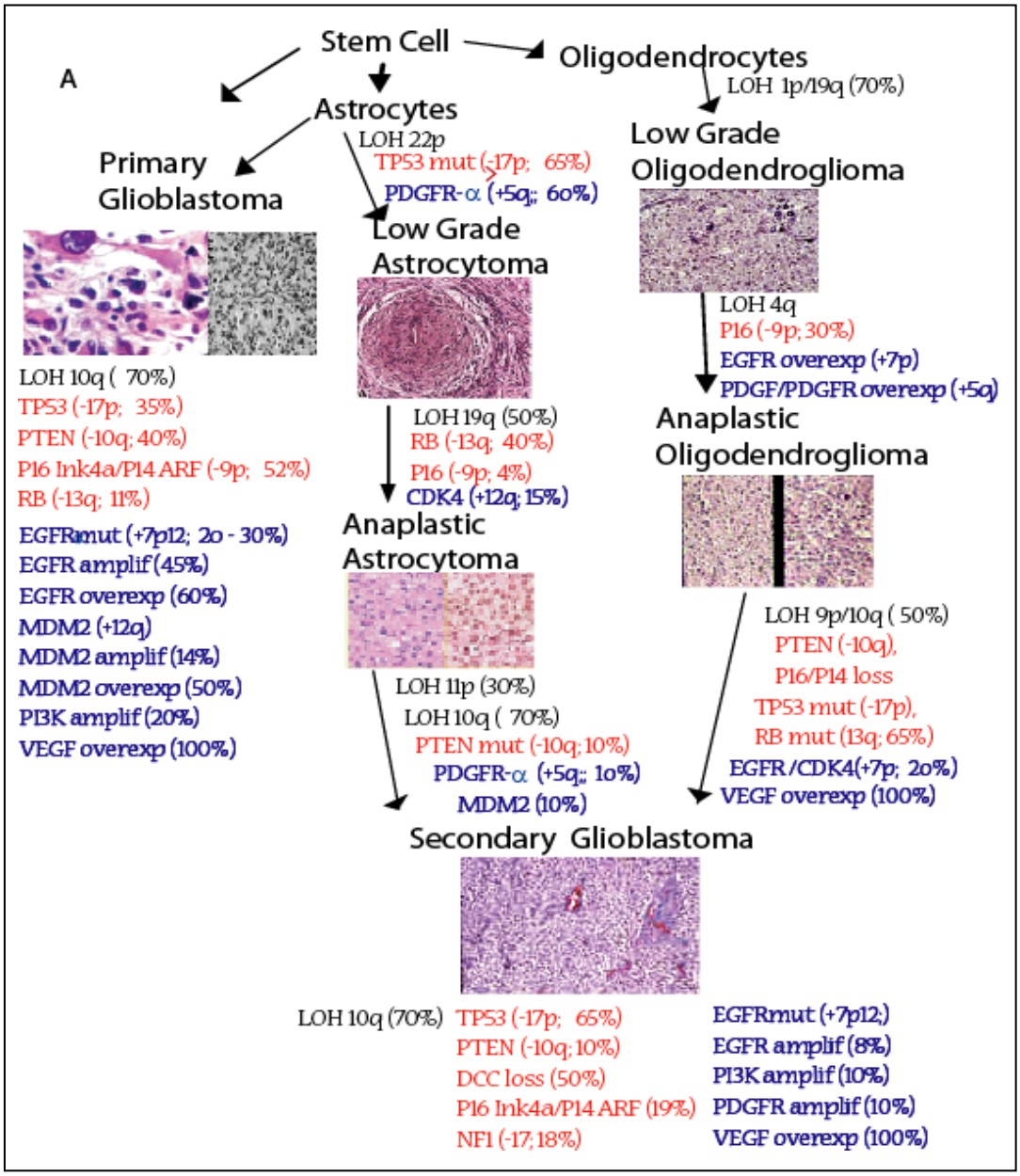
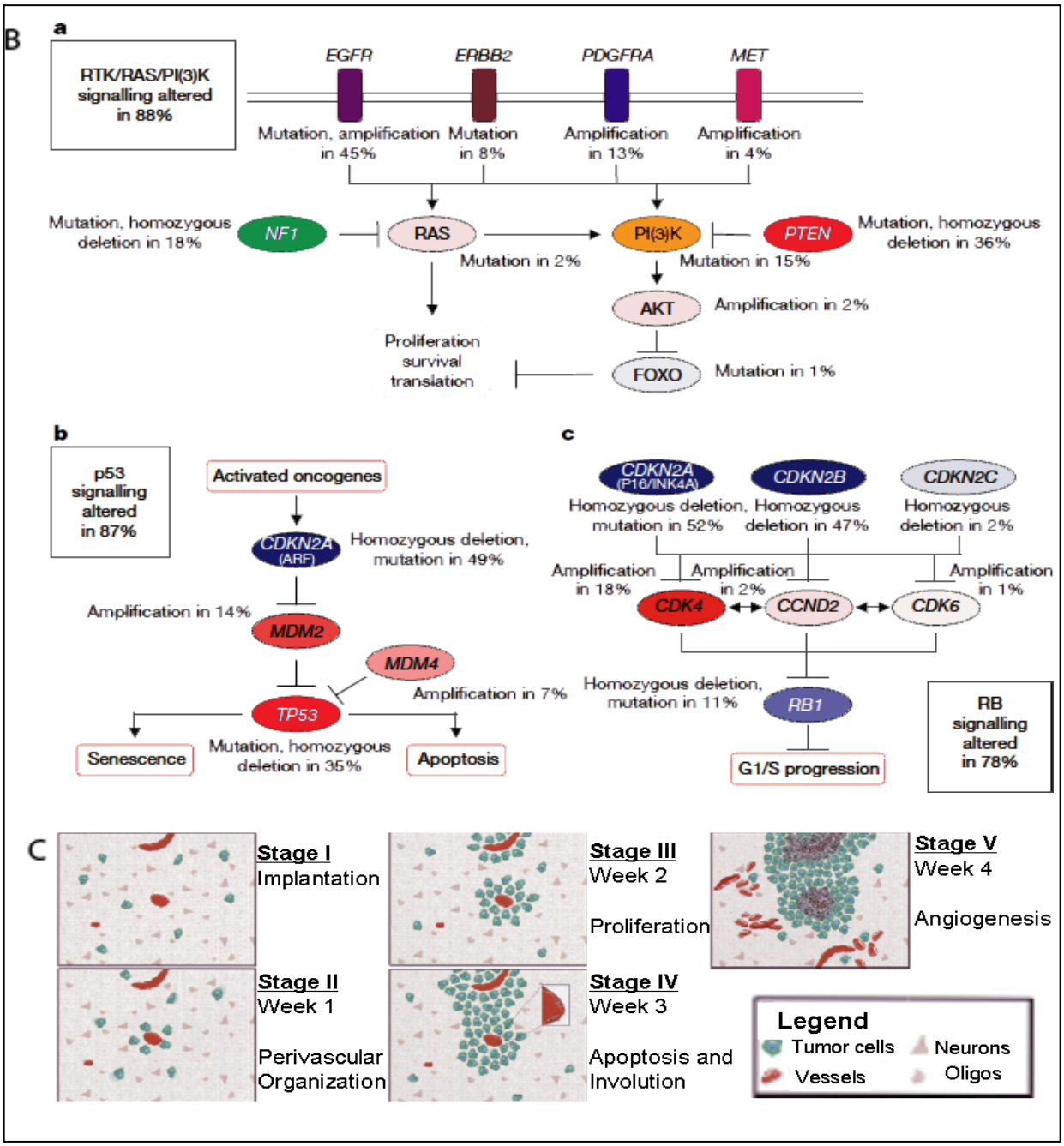


Figure 3 Genetic profile by grade of malignancy, pathways and stages of growth involved in the development of GBMs [Modified from 19, 20, 21, 22]. A) Relationships between histopathology and the molecular lesions that lead to the formation of primary and secondary GBMs. Genomic rearrangements are shown in black; tumor suppressor genes that are either lost or inactivated by mutation are shown in red and proto-oncogenes /growth-promoting genes activated by mutation or overexpressed are in blue.



B) Frequent genetic alterations in three critical signaling pathways. a-c> Primary sequence alterations and significant copy number changes for components of the RTK/RAS/PI(3)K(a), p53(b) and RB(c) signaling pathways are shown. For each altered component of a particular pathway, the nature of the alteration and the percentage of tumors affected are indicated. Boxes contain the final percentages of GBMs with alterations in at least one known component gene of the designated pathway. C) The five stages followed by GBMs growth in the brain.

The molecular profile of primary and secondary GBMs related with their final outcome shows that: A) 95% of cases belong to primary GBM. The genetic alterations appear to be mutually exclusive in both cases, especially with respect to the RB, EGFR, TP53, MDM2 and chromosomes 1p and 19q. B) The genetic alterations mainly involved with poor prognosis and survival of the patient are the homozygous deletion of the CDKN2, INK4α and ARF genes in association with the EGFR and MDM2 genes amplification (related with higher proliferative indices). The loss of heterozygosity in 9p, 10q and p16 deletions are also observed (Table 2) [3, 10, 16, 23, 24].

Table 2 Genetic characteristics of primary GBMs, secondary GBMs and genetic factors affecting theirs final outcome.

A

Glioma type	Gain of function	Loss of function	Other meaningful characteristics
Primary Glioblastoma	EGFR amp (7p; 40%) EGFR overexp (80%) MDM2 overexp (12, 5%) PDGFA overexp BCL2L overexp (12; 95%) Telomerase activity (20%)	LOH 9p P16CDKN2 (9, 35%) PTEN mut (10, 30%) RB (13%) TP53 (10%)	Mean age 62 years old Survival 9 to 12 months Sex ratio (M:F) 1,4 95 % of cases
Secondary Glioblastoma	PDGF/PDGFR RAS/MYC/CDK VEGF Telomerase activity (70%) EGFR (8%)	LOH 1p, 9p, 13q, 19q Loss of chr10q PTEN (5%) P16CDKN2 (5%) TP53 (65%)	Mean age 45 years old Survival 12 to 15 months Sex ratio (M:F) 0,8 5 % of cases

B

Glioma type	Gain of function	Loss of function	Other meaningful characteristics
1 / 3 of all gliomas	Chr17p Δ p53 Chr19q	Chr5q PDGF – α	Young patients; better prognosis
1 / 3 of all gliomas	Ch10 PTEN	Chr7 EGFR FGF	Recur more quickly
1 / 3 of all gliomas	Chr1p	Chr17 NF1	Affect the chemotherapy outcome

*Underlined are the genetic changes for oligodendrogliomas-derived tumors. Modified from [21, 23, 24].

Primary GBM occurs most commonly in elderly patients (mean age 62 years), while secondary GBM afflicts younger population (mean age 45 years) [20-22]. Some tumors are born being malignant (primary GBM) while in other cases, benign tumors become malignant, but the reality of this disease is that intracranial tumors, whether low or high grade, are all devastating and lethal. That is why it is important to study GBMs biology and find different ways to control this disease.

I.2.2 Glioblastoma's vasculature

Glioblastomas, the most frequent and malignant brain tumors, are known for their ability to trigger the formation of blood vessels by the process of angiogenesis [25]. This process occurs as an orderly series of events: release of angiogenic growth factors, activation of endothelial cell receptors of nearby blood vessels, signal falls are sent from the cell's surface to the nucleus, production of enzymes to dissolve the basement membrane of nearby preexisting blood vessels, the endothelial cells begin to divide (proliferate) and migrate out through the dissolved holes of the existing vessel towards the diseased tissue (tumor). The last events include specialized molecules called adhesion molecules called integrins (avb3, avb5) serve as grappling hooks to help pull the sprouting new blood vessel sprout forward. Additional enzymes (matrix metalloproteinases or MMP) are produced to dissolve the tissue in front of the sprouting vessel tip in order to accommodate it. As the vessel extends, the tissue is remolded around the vessel. Sprouting endothelial cells roll up to form a blood vessel tube. Individual blood vessel tubes connect to form blood vessel loops that can circulate blood. Finally, newly formed blood vessel tubes are stabilized by specialized muscle cells (smooth muscle cells, pericytes) that provide structural support. Blood flow then begins [26].

Although it is established that angiogenesis can be a critical determinant of tumor growth in certain conditions, accumulating evidence suggests that malignant tumors require the formation of new blood vessels to grow beyond a certain size when implanted into avascular settings or artificially created sites (example, by subcutaneous injection). However, recent observations indicate that tumors in more natural settings do not need new blood vessels, particularly when they grow in highly vascularized tissues such as the brain. In fact, the processes of vessel co-option and new vessel formation (angiogenesis) are not mutually exclusive, but can occur concurrently depending on the pathophysiological conditions [27, 28] (Figure 4). Malignant gliomas grow by exploiting the existing vasculature and angiogenesis is a relatively late process that occurs mainly at the tumor margin after a phase of vascular regression. It has been proposed that the process of co-option begins with the accumulation and proliferation of glioma cells around the vasculature, disrupting the association of endothelial cells with the basement membrane and astrocytic foot processes [29] (Figure 5).

Co-opted vessels undergo structural and functional changes that promote edema formation and tumor growth. It has been proposed that these changes are initiated by the accumulation and proliferation of tumor cells around the vasculature, disrupting the association of endothelial cells with the basement membrane and astrocytic foot processes. As a consequence, endothelial cells express angiogenic factors that further destabilize the vasculature, as manifested by an increase in vessel caliber and permeability. Since it is an early and continuous process, vessel cooption may prove to be a better therapeutic target than angiogenesis, which occurs later in response to ischemia, hemorrhage, or necrosis [29, 30].

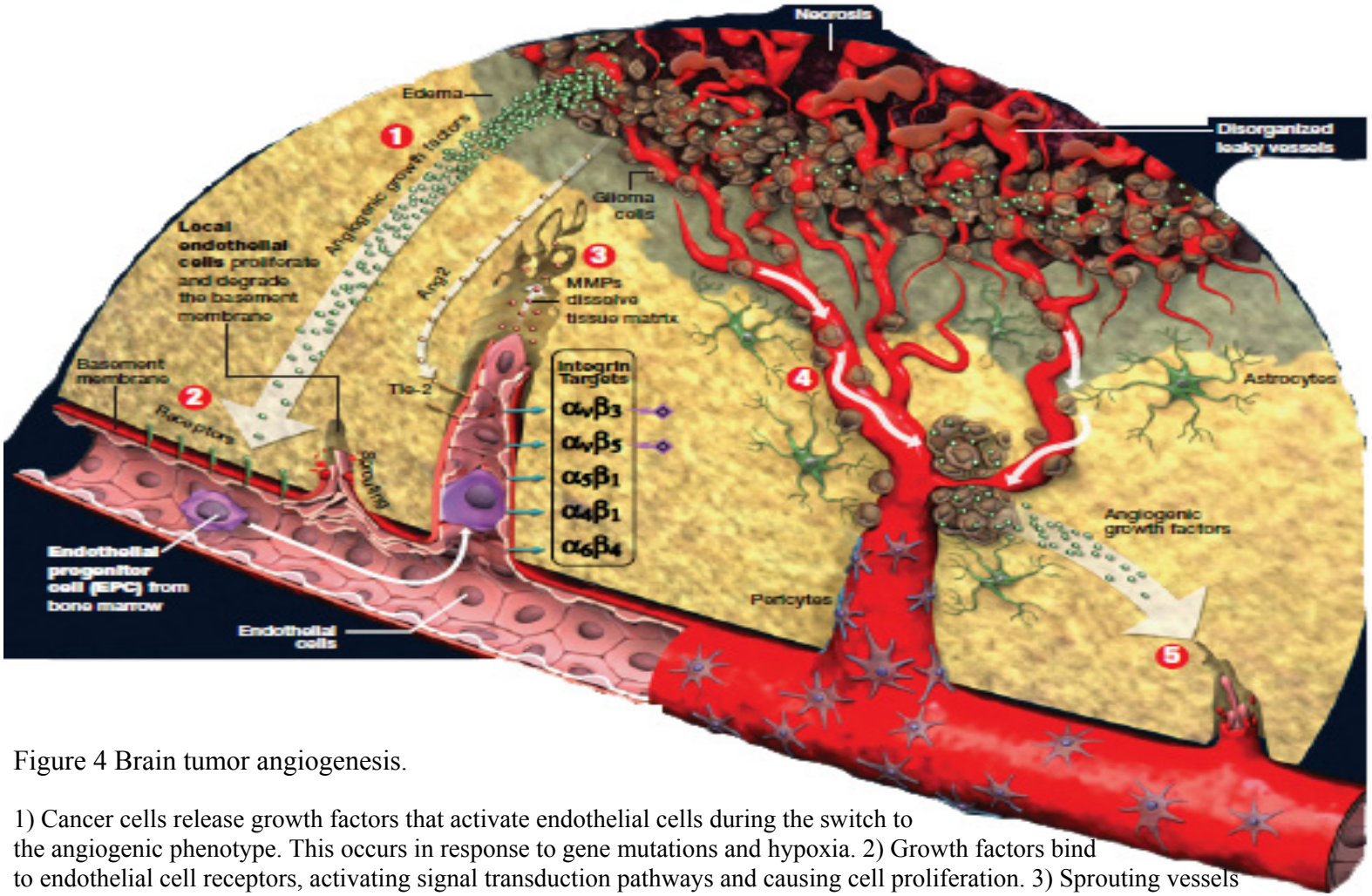


Figure 4 Brain tumor angiogenesis.

1) Cancer cells release growth factors that activate endothelial cells during the switch to the angiogenic phenotype. This occurs in response to gene mutations and hypoxia. 2) Growth factors bind to endothelial cell receptors, activating signal transduction pathways and causing cell proliferation. 3) Sprouting vessels secrete matrix metalloproteinases (MMPs) and migrate towards the tumor using specific αβ integrins. 4) Glioma cells migrate away from the primary tumor along the outside of host vessels. Proliferating tumor cells surround and 'co-opt' host vasculature. 5) Once the glioma cell clusters reach a sufficient size, they begin recruiting their own vasculature and a new tumor forms [31, 32].

GBM cells do not generally need new vessels to progress, particularly at the invasive front, where they simply exploit the pre-existing vasculature [29, 33]. In fact, GBM cells mainly migrate coopting the vascular network in the brain, but these cells are also able to use white matter tracts, the glia limitans externa and the subependyma [34, 35]. Recent studies of the *in situ* biology of invasive GBM cells (palisading cells) suggests the activation of genetic and cellular programs (Figure 4) that allow them to coopt the normal vasculature in order to invade the neighboring tissue [28, 36-39].

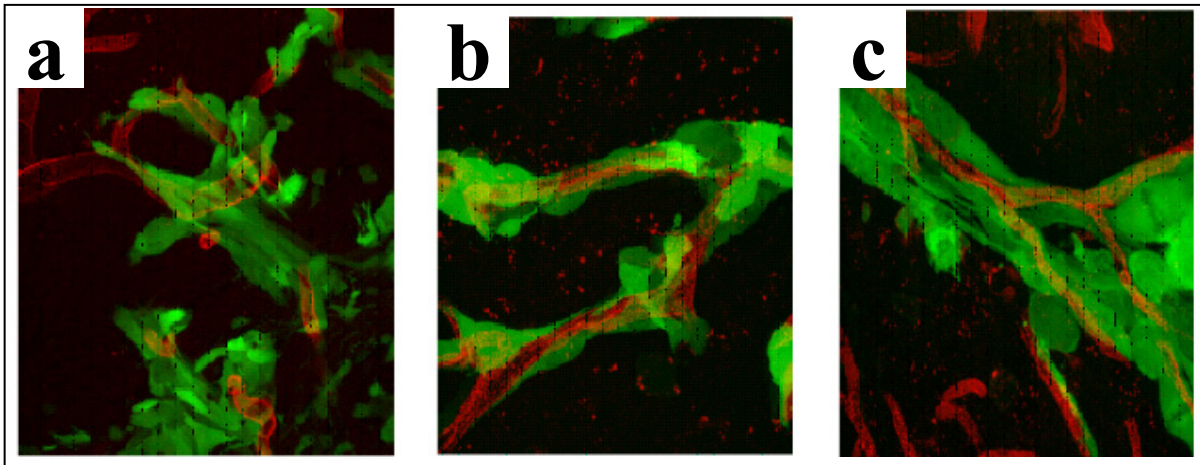


Figure 5 Perivascular organization of three glioma cells lines (green) co-opting blood vessels (red) in our glioma mice model. a) GL261, b) LN229, c) SKY-1

Accordingly, Shiffer reported that tumor growth requires neovascularization while infiltrating tumor likely utilizes pre-existing vessels [40, 41]. Retrovirus mediated expression of a dominant negative VEGFR-2 mutant suppresses the growth of GBM as well as other tumor cell lines *in vivo* [42]. The formation of new vessels requires the coordination of angiogenic and antiangiogenic factors that regulate the induction of vascular discontinuity, endothelial cell proliferation and migration, and structural reorganization of new vasculature [43, 44].

I.3 Tumor blood vessel (TBV) characteristics

To understand the role of blood vessels in GBM growth, it is crucial to clarify the interaction of tumor cells and blood vessel cells. Brain tumors have heterogeneity of microvascular distribution, diameter and glomeruloid vessels. Tumor blood vessels are modified accordingly to the grade of the tumor (lower grade astrocytomas incorporate pre-existing vessels, while GBMs develop new vessels) and their location inside the tumor (Table 3) [41, 43, 45]. Tumor vessels are extremely permeable due to the presence of fenestrae, transcellular holes, and the lack of a complete basement membrane [30, 46, 47].

Table 3 Tumor blood vessel densities according to grade of malignancy.
Modified from [41, 43, 45].

Brain tumors		Microvessel density	
Glioma grade II	Gangliogliomas, astrocytomas, Ependymomas	10-83 microvessels / 200 x field	
Glioma grade III – IV	Glioblastoma, anaplastic ependymoma, cerebellar astrocytoma	83–179 microvessels / 200 x field	
Medulloblastomas		23–47	Two different studies
		9–40	

In order to understand the relationship between tumors and their vasculature and identify some potential therapeutic targets, the analysis of the transcriptome profile of different glioma cell lines was done. Additionally, the correlation with cell characteristics like migration in a perivascular fashion and the ability to induce vascular changes was established. Among targets with interesting therapeutic potential are *Angiopoietin 3* and *Tie2 receptor*.

I.4 Angiopoietins

Besides the VEGFs, angiopoietins are the only other known growth factors specific to the vascular endothelial cells implied in vascular development and angiogenesis. At the same time, they are highly expressed in the hypoxic regions of GBMs, therefore our interest for this family of proteins [48, 49].

All angiopoietins bind with similar affinity to an endothelial cell-specific tyrosine-protein kinase receptor and activate it by inducing its tyrosine phosphorylation [50]. In general, angiopoietins are secreted glycoproteins playing a critical role in mediating reciprocal interactions between the endothelium and surrounding matrix and mesenchyma. They contribute to blood vessel maturation and stability, and may be involved in early development of the heart [51, Table 4].

The angiopoietin family includes the naturally occurring agonist Angiopoietin 1 (Angpt1) and the antagonist Angiopoietin 2 (Angpt2). The *Angpt1* gene encodes 498 amino acid residues, including an amino-terminal secretion signal sequence. The *Angpt2* gene encodes a protein of 496 amino acid residues, with a secretion signal peptide. *Angpt1* and *Angpt2* are 60% identical [52-54]. More recently, two new members have been described: Angiopoietin-3 (Angpt3) in mouse, which acts as an antagonist and Angiopoietin-4 (Angpt4) in humans, acting as an agonist. Although *Angpt3* and *Angpt4* are structurally more diverged from each other than the mouse and human versions of *Angpt1* and *Angpt2*, they represent the mouse and human counterparts of the same gene locus [55].

Angpt1 is widely expressed although in small amounts in the heart and liver [53]. It plays a crucial role in mediating reciprocal interactions between the endothelium and surrounding matrix and mesenchyma [56], and a role in the maintenance of normal vasculature and Tie2 activity [57]. *Angpt1* mRNA is found in tumor cells and *Angpt2* mRNA is observed in the endothelial cells of a subset of GBM blood vessels. Strong expression of *Angpt2* originates from small capillaries with few periendothelial support cells, whereas larger GBM vessels with the same characteristics show little or no expression [58]. In adult mice and human tissues, Angpt2 is detectable only in ovary, placenta, and uterus which are the three predominant sites of vascular remodeling. Transgenic over expression of Angpt2 in

vascular structures results in disrupted blood vessels [53]. In fetal liver, Angpt2 is not present in all vascular structures, but is detected in cells at, or close to, the lumen of hepatic vessels, in cells which are probably endothelial cells or pericytes [59]. In NIH-3T3 cells ectopically expressing *Tie2* receptors, Angpt2 is equivalent to Angpt1 in inducing the phosphorylation of the receptor. It seems that Angpt2 acts as an antagonist for Tie2 in the context of the endothelial cells, but not in other cell types [53].

Angpt1 may function in concert with VEGF to stimulate vessel sprout. Angpt2, which becomes upregulated in endothelial cells of angiogenic capillary sprouts, may disrupt the interactions between endothelial cells and pericytes, thus sensitizing the endothelium to the mitogenic and chemotactic signals secreted by the tumor [60]. In contrast, Angpt2 promotes angiogenesis in the presence of VEGF by inhibition of the stabilizing effect of Angpt1 and the subsequent loosening of the contacts between endothelial and periendothelial cells [61].

Angpt3 mRNA is expressed in lung, liver, cerebellum, and heart in response to hypoxia in rats [62], whereas *Angpt4* transcript is expressed at high levels only in human lung [55]. Angpt1/Angpt4 binds and induces the tyrosine phosphorylation of Tie2 promoting angiogenic remodeling as well as vessel maturation and stabilization [54]. Angpt2 and Angpt3 bind Tie2, but do not activate it [55], thereby blocking Angpt1/Angpt4 activity in endothelial cells. Recently, it was demonstrated that Angpt3 and Angpt4 are agonists of Tie2, but mouse Angpt3 has strong activity only on endothelial cells of its own species [63].

Table 4. Summary of angiopoietins characteristics.

See text for references.

Action	Molecule	Expressed	Role	Others specifications
Agonist*1	Angpt1	Basal conditions (Heart, liver)	Inhibits apoptosis of endothelial cells; mediate stabilization and maturation of newly formed blood vessels via assembling of periendothelial cells such as pericytes or smooth muscle cells into the vascular wall	Maintenance of normal vasculature and Tie2 activity
	Angpt4	Lung	In concert with VEGF promotes angiogenesis	Human counterpart
Antagonist*2	Angpt2	Pathological conditions (mRNA of endothelial cells of tumor blood vessels)	Vascular remodeling, Angpt2 antagonizes the stabilizing effect of Angpt1	Overexpressed in endothelial cells of disrupted blood vessels
	Angpt3	Basal conditions (lung, liver, cerebellum, heart)	Inhibits the ability of carcinomas to metastasize in the lungs	Murine counterpart Overexpressed in response to hypoxia

*1 Activate Tie2 phosphorylation
*2 Do not activate Tie2

The signal transduction pathways followed by the vascular endothelial growth factor (VEGF) and angiopoietins in order to activate the cellular signal transduction to promote gene expression, DNA synthesis, migration and survival of the endothelial cells are shown in figure 6. These signal falls stimulate angiogenesis, vascular tone and permeability of blood vessels [64, 65].

Actual antiangiogenic research has shown that the interference of angiogenesis holds up migration of gliomas making anti-angiogenic therapies also anti-migratory. In fact, overexpression of *Angpt3* inhibits the ability of carcinomas to metastasize in the lungs, an organ in which Angpt3 induces Tie2 phosphorylation and activation [66]. Also, the administration of sTie2 inhibits both growth and metastasis of carcinoma and melanoma cells in mice by exerting antiangiogenic activity [64].

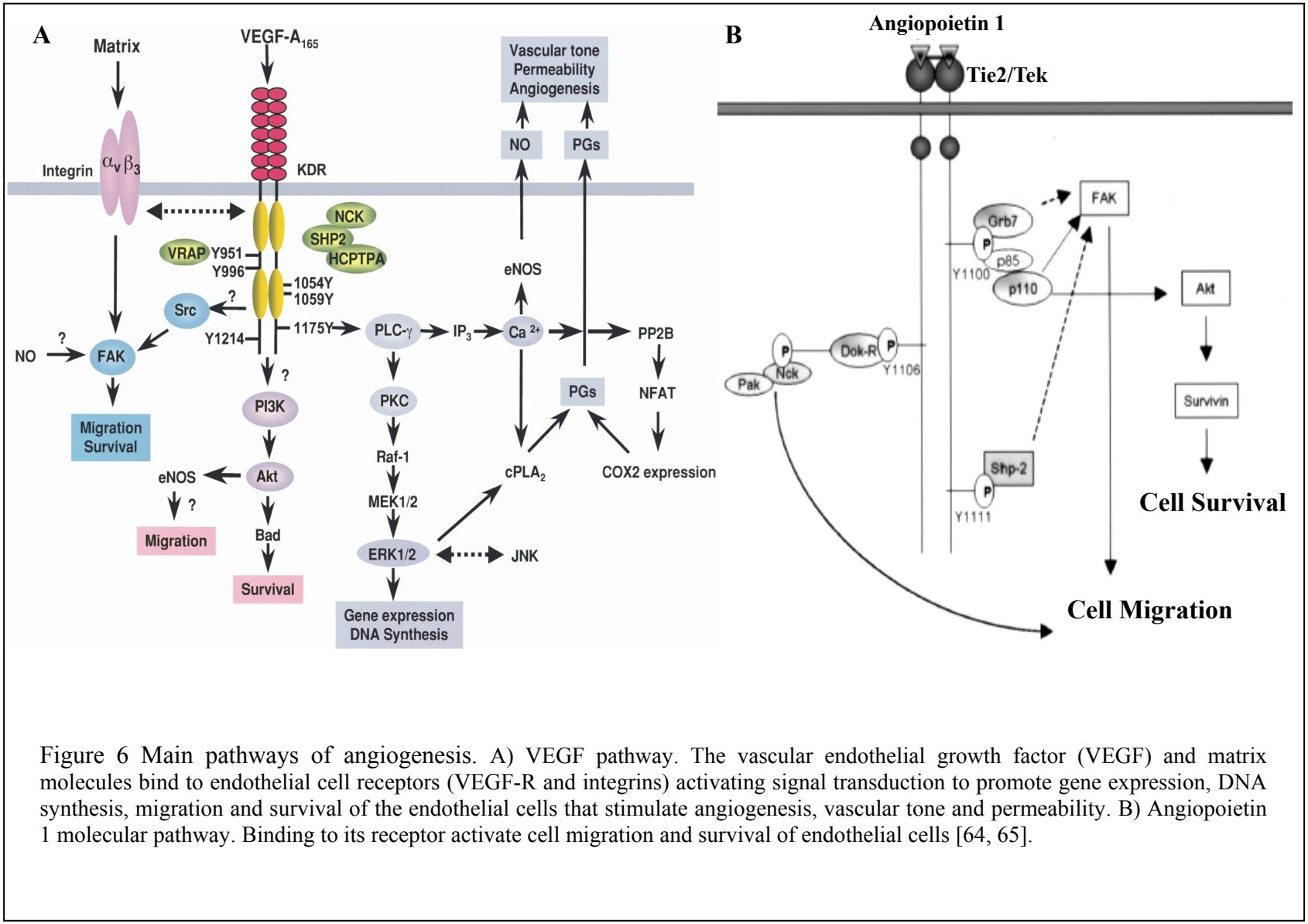


Figure 6 Main pathways of angiogenesis. A) VEGF pathway. The vascular endothelial growth factor (VEGF) and matrix molecules bind to endothelial cell receptors (VEGF-R and integrins) activating signal transduction to promote gene expression, DNA synthesis, migration and survival of the endothelial cells that stimulate angiogenesis, vascular tone and permeability. B) Angiopoietin 1 molecular pathway. Binding to its receptor activate cell migration and survival of endothelial cells [64, 65].

Although the molecular mechanisms involved in vessel cooption are largely unknown, recent evidence points to a central role of angiopoietin-2 [29, 67]. Strongly expressed in human and experimental gliomas, Angpt2 could not only cause a breakdown of the blood-brain barrier, but also contribute to glioma growth by facilitating the process of cooption [68]. This possibility has recently been addressed in our laboratory by treating glioma-bearing mice with an anti-Angpt2 peptide-Fc fusion protein called L1-10, which has recently been used with success to inhibit the development of epidermoid and colorectal tumor xenografts, reduced glioma volume by 50% increasing the survival of glioma-bearing mice. The later antitumor effect was due to a decrease in tumor vascular density (9%), reduced tumor vessel diameter by 29% and a small reduction in macrophages (17%). L1-10 treatment supports an important role for Angpt2 in vessel cooption and glioma growth [69, 70] (Figure 7).

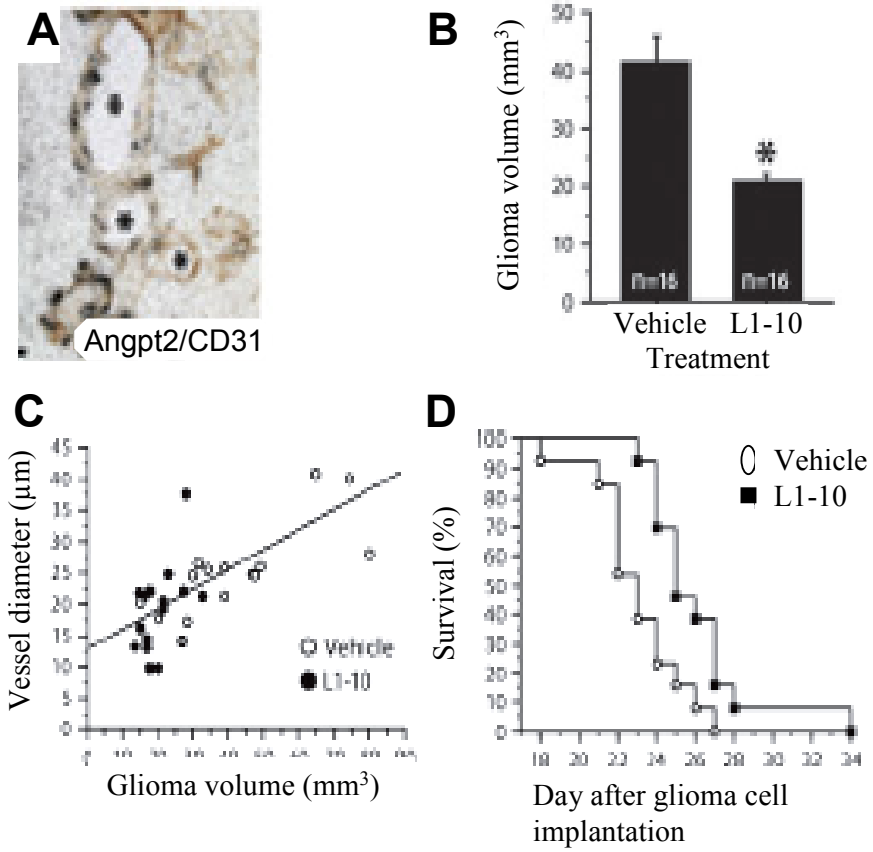


Figure 7. Specific neutralization of Angpt2 with L1-10 reduces glioma growth and vascular enlargement. A) Double labeling for Angpt2 mRNA (*in situ* hybridization, black grains) and CD31 (immunohistrochemistry, brown). *Blood vessel lumen. B) Volumetric analysis showed a 50% decrease in glioma volume in L1-10 treated mice. *Wilcoxon rank-sum test, P=0.0002. Data show the mean_SE. C) Tumor vessel diameter was reduced by 29% in L1-10-treated mice and correlated positively with glioma volume (Pearson correlation, P=0.0002, R=0.61). *Wilcoxon rank-sum test, P=0.0053. Each data point represents a mouse treated with L1-10 or vehicle. D) Kaplan-Meier curves showing a modest increase in the probability of survival after treatment with L1-10. Log-rank test, P=0.005 (n=13 per group) [70].

II Treatment of glioblastomas

II.1 Current treatments

Treatment of tumors is based on their type and location. As noted above, all gliomas are potential GBMs, but the location of the tumor could promote the early detection of the tumor and therapeutics that will block the natural course of the disease. Current treatments include removal of the tumor and surrounding tissue (if not committed to vital biological functions of the patient), chemotherapy and radiotherapy combined with dexamethasone, an anti-inflammatory drug, to regulate side effects such as inflammation, edema, osteoporosis due to cranial pressure, and so on. The goal of these treatments is to prolong survival and quality of life of the patients. The average survival after diagnosis has not been altered in the last 30 years, being 13 months and only 3 months in case of recurrence [71].

The treatment of GBM is case specific and it is difficult to create a single formula to cure it. Each tumor is different and contains different kinds of cells that may respond differently to each method of treatment. The goal of the actual therapy is to use as many combinations of treatments as necessary in order to wipe out as many tumor cells as possible [72]. Surgery is performed to remove as much of the tumor as possible [73]. Following surgical removal, adult patients receive one or multiple forms of radiation therapy. Computerized tomography scans and magnetic resonance imaging are made immediately after surgery, and then at regular intervals to ensure an appropriate follow-up. Although chemotherapy was found to be ineffective for treating GBMs, it is effective on oligodendrogliomas. In the case of early prognosis of glioma, a simple bipolar cautery and suction (ultrasonic aspirator) are usually sufficient for resection. A second operation may be necessary to remove re-growth of tumor tissue [35, 71], which if often located within 1 to 3 cm of the resection margin and accounts for the fatal outcome of the disease [74]. In terms of invasion, we can say that low grade glioma grows 10 times slower than high grade glioma [75].

To increase quality of life and survival of patients affected by this deadly disease, more effective treatments are needed. That is why it is important to increase our understanding (at the molecular level) of the biological mechanisms regulating development, energy input and invasion / infiltration of brain tumors. The control of the genes required for the growth, survival, and infiltration of these malignancies makes gene therapy a promising alternative treatment [76, 77].

II.2 Future treatments

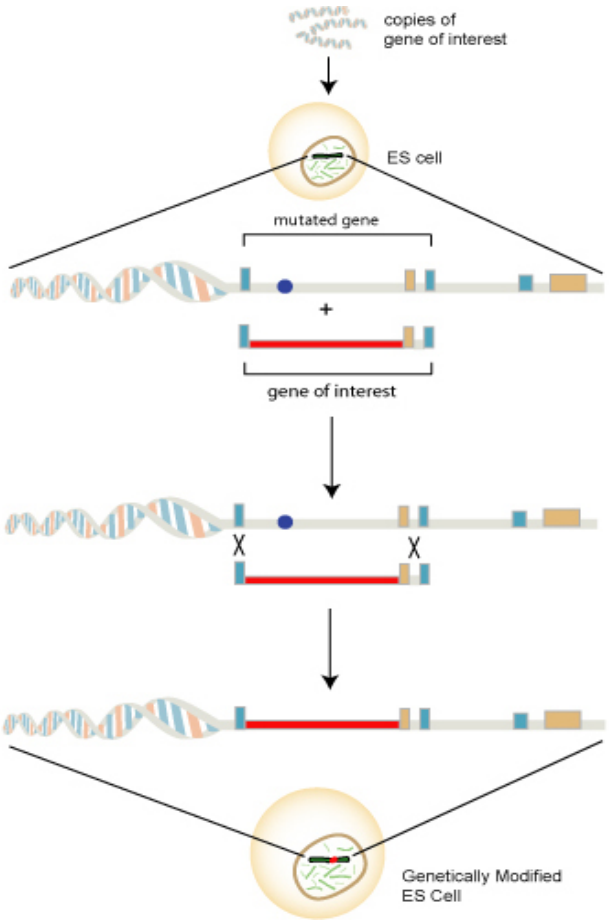
The development of novel treatments requires improved pharmacological approaches that increase and prolong responses to therapy and increase the cure rate with acceptable toxicity.

II.2.1 Gene therapy

The basic idea of using gene as therapeutic tool came from the 60's when Tatum, Lederberg, Anderson *et al.* figured out that specific genes can be manipulated and used to treat acquired and hereditary human diseases [78]. A large number of gene therapeutic strategies have been proposed and carried out, but the first gene therapy started in 1990 and it was proposed to treat adenosine deaminase (ADA) deficient patients. Target diseases extended from single factorial disorders, such as congenital metabolic diseases to multifactorial diseases, such as cancer.

The vehicles or vectors used to deliver the gene(s) inside the target cell are classified in viral vectors or non viral vectors. The basic requirements of all vehicles used in gene therapy, besides sustainable and safe gene expression, are: high DNA capacity, low host toxicity, transgene expression control and easy manufacture. All vectors or vector-producer cells are directly injected (*in vivo* or *in utero* platforms) or indirectly (*ex vivo* platform) (Figure 9 and Annex II).

A



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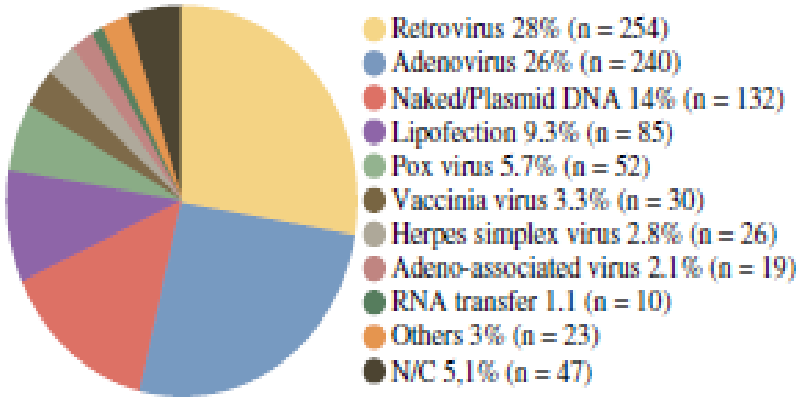


Others : naked DNA application, high-pressure injection, liposomal delivery, and cationic cholesterol-containing liposomal constructs

Figure 8. Gene therapy concept and vectors. A) Diagram showing the transfer of genetic material in order to create a stem cell genetically modified. B) Gene therapy vectors used in clinical trials. N/C= not communicated. DOE USA online.

Cancer gene therapies are classified according to the molecules targeted in immunological approaches and molecular therapies. Immunological therapies are immuno-stimulant genes and vaccines (cell and gene vaccines). Molecular targeted therapies include suicide genes, tumor suppressor genes and anti-oncogenes (antisense and antigene oligonucleotides). In this work we did choose the suicide gene strategy, which is described later in the treatment sub-chapter.

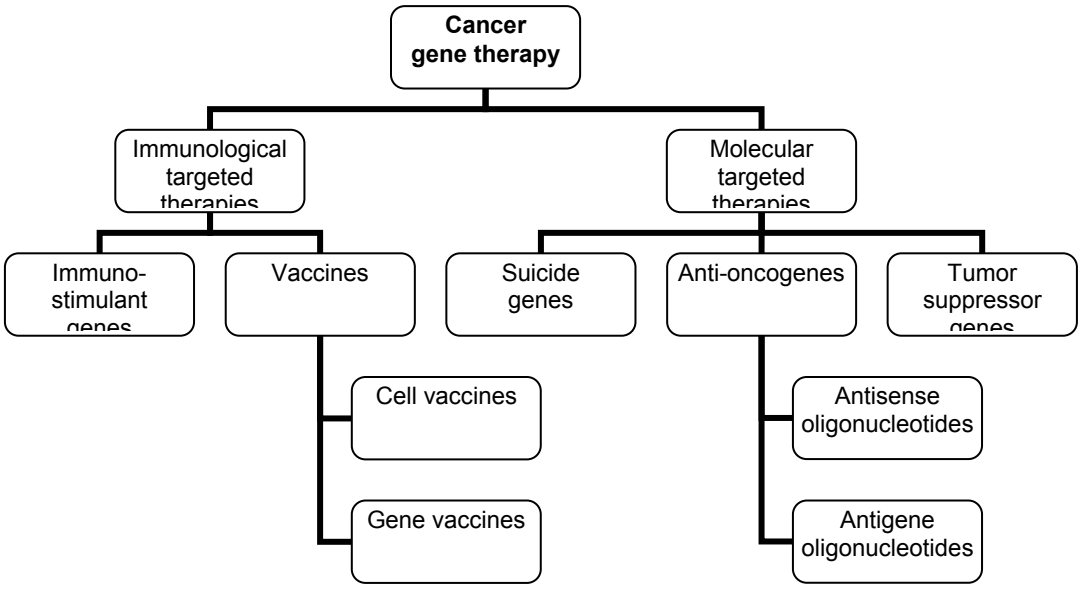


Figure 9. Summary of the most common current cancer gene therapy approaches. (Further information [79]).

The relative ineffectiveness in achieving long-term survival and the toxicities associated with conventional treatment for GBM have prompted investigators to look at gene therapy as an alternative treatment. Gene therapy also offers the possibility of rendering current therapeutic approaches more effective by improving the selectivity and safety of treatment. Treatment strategies include pro drug activation within a tumor, delivery of a secreted fusion protein in combination with a targeting ligand coupled to a toxin or enzyme, and introduction of radiosensitizing agents directly into the tumor. Transfected cells or vectors can be directly injected into the tumor bed, and tumor cells can be used to develop an anti-tumor vaccine. Finally, gene therapy can be designed to act directly on tumor cells to block their growth, inhibit angiogenesis, prevent further tumor extension, or stimulate immune-mediated attack of tumor cells.

II.2.1.1 Characteristics of lentiviral vectors for gene therapy

Lentiviral vectors are members of the retrovirus family. These vectors can transduce nondividing as well as dividing cells. Many lentiviral vectors are derived from the human immunodeficiency virus (VIH) and are modified such that less than 5% of the parental genome is retained in the vector and less than 25% of the viral genome is incorporated into packaging constructs. These small percentages minimize the probability of replication-competent revertants. Lentiviral vectors have been proposed for treating a wide range of diseases such as hematopoietic disorders and CNS diseases.

Nonetheless, lentiviral vectors are powerful tools for gene transfer, but their use in humans raises biosafety concerns. For example, they can potentially cause cancer by insertional mutagenesis or by carrying a transgene that provides cells with a growth advantage. In addition, as they permanently integrate into the host genome, the transgene could become toxic if transduced cells proliferate and disseminate to other organs in an unwanted manner.

The main advantage of lentiviral vectors is the stable expression of the transgene in non-dividing cells as well as low antivector immunity (Annex II). In self-inactivating lentiviral vectors (SIN), the packaging signal required for vector mobilization is eliminated to improve their safety. Another improvement aiming the control of the site of viral insertion is found to decrease the efficiency of transduction and reduce the possible target diseases treated by lentiviral vectors [80]. Nevertheless, the main disadvantage of lentiviral vectors is the uncontrolled effect of insertional mutagenesis [81]. In 2003, leukemia was provoked in three children treated for severe combined immunodeficiency disease (SCID). Lymphoma has also been linked to the use of this kind of vectors [82] showing the need of more suitable and safe vectors.

Alternatives for the classic suicide gene therapy were studied aiming to improve the lentiviral vector by making it safe by autodestruction or suicide. In Table 4, the main characteristics of the suicide gene system, advantages, disadvantages, mechanism of function and schedules usually used in animal models are shown.

II.2.1.2 Virus-directed suicide gene therapy (TK/GCV)

The general principle for suicide gene therapy systems is the negative selection of target cells for their elimination. Inoculation of one susceptibility factor (suicide gene) into the target cells make them capable of transforming the prodrug, otherwise benign chemical compound, into a cytotoxic drug. Some systems inhibit DNA synthesis, RNA processing and/or directly block translation of proteins (Table 4) [83].

The most studied suicide gene so far is the herpes simplex virus thymidine kinase gene (HSV*tk*). HSV*tk* was the first suicide gene that was demonstrated to confer chemosensitivity to tumor cells by the phosphorylation of the prodrug ganciclovir (GCV), a guanosine analogue [84]. Ganciclovir monophosphate is further phosphorylated by normal cellular kinases to the toxic triphosphate form. Ganciclovir triphosphate (GCV-PPP) acts as a DNA polymerase inhibitor and a chain terminator, leading to the death of prodrug-treated proliferating HSV*tk* expressing cells and neighbouring cells via the transport of GCV-PPP through gap junctions (bystander effect) [85-87].

The use of viral vectors to express HSV*tk* in rodents and its clinical application in humans is summarized in Table 5. Animal studies that have used this system have shown inhibition of tumor growth proliferation. However, human clinical trials have been partially effective, showing a reduction in the tumour size in some of the patients treated. The limitations of this methodology are related to GCV-resistant relapses associated with post insertional alterations of transgene structure, loss of the entire transgene, short half life and transgene silencing occurred in another clone [88].

The advantages of ganciclovir therapy are its large therapeutic index, the bystander effect with only 10% of transduced cells, the induction of apoptosis and cellular arrest as well as triggered infiltration of CD4 and CD8 positive cells, and macrophages. It has also been demonstrated the stimulation of HMGB production is promoting the activation of cytotoxic T lymphocytes [89].

Lentiviral gene-suicide gene therapy targets proliferating and non-proliferating cells, inducing a limited inflammatory response. The presence of the herpes simplex virus thymidine kinase (HSV*tk*) gene in transduced cells will kill all transduced cell [90] and nearby cells that are not expressing HSV*tk* by bystander effect [91].

Different anticancer therapies have used tumour suppressor genes, suicide genes, anti-angiogenic genes, anti-sense technology and stimulation of immunological genes [14, 90]. Improving the efficiency of gene delivery and avoiding the toxic side effects caused by viral vectors is critical to the future success of gene therapy.

Table 4 Characteristics of suicide gene therapy systems.

Suicide Gene Therapy System	Advantages	Disadvantages	Mechanism of Action
TK/GCV[*1] For references see Table 5.	⌖ Large therapeutic index. ⌖ Bystander effect. ⌖ Transduced cells are rendered more sensitive to chemotherapy and radiotherapy. ⌖ Apoptosis independent of p53 or death receptors (TNF, CD95). ⌖ Decrease Bcl-2 levels or promotion of its phosphorylation destabilizing microtubuli and inducing a late S and G2/M phase cellular arrest. ⌖ Alteration of the immune cell response (CD4, CD8 and macrophages). ⌖ Stimulation of HMGB production promoting the activation of cytoxic T lymphocytes.	⌖ Glioma cells resistance after longer exposition to high doses of GCV. ⌖ Bystander effect dependent of gap junctions. ⌖ Kill only transduced cells in proliferation.	⌖ Triggering tumor cell death by causing chain termination and single strand breaks upon incorporation into DNA.
CD/5FC[*2] Cancer Gene Therapy (2000) 7:74-82 J Neuro-oncology (2004) 66:117-127	⌖ Large bystander effect (just 2-4% of transduced cells independent of cell-to-cell contact). ⌖ Suitable for brain tumors because it can readily cross the blood brain barrier (BBB). ⌖ Apoptosis (independent of p53 or death receptors (TNF, CD95). ⌖ Toxicity for both proliferating and non proliferating cells. ⌖ FACS shows that 5-FC-treated cells accumulate in the G0/G1 phase. ⌖ Increase GCV mediated citotoxicity.	⌖ Low therapeutic index (prodrug concentration is 10 times higher than GCV to obtain similar results). ⌖ Toxic to proliferating and non-proliferating cells. ⌖ rAdV-CD shows areas of necrosis encircled by apoptotic cells, demyelination and gliosis within areas of normal brain. ⌖ High doses cause edema, meningitis, demyelination, hydrocephalus and necrosis.	⌖ Trigger tumor cell death through inhibition of thymidylate synthase, depletion of dTTP pools, DNA double-strand breaks and inhibition of RNA nuclear processing.

CD/TK[*3] Oncogene (2005) 24:1231 Cancer Res (2006) 66(6): 3230-3237 Chinese medical journal (2004) 117:1464-70 Neurosurgery (2000) 47:931	⌖ Large synergy effect. ⌖ Both prodrugs engage the apoptotic mitochondrial pathway. ⌖ When applied to rat vascular smooth muscle cells using 0.5 µg/mL of GCV and 50 µg/mL of 5FC, the cellular survival rate is as follows: CD 46 % TK 37 % CD-TK 9 %	⌖ Synergy phenomenon is increased when sequential treatment is followed.	⌖ Tumor cell death.
PNP[*4] Cancer Research (2004) 64:6610-6615 Cancer Gene Therapy (2003) 10:23-29	⌖ Excellent antitumor activity. ⌖ Different prodrugs might be used with this enzyme. ⌖ Toxicity to both proliferating and non proliferating cells. ⌖ Bystander effect with just 2-5% of transduced cells independent of cell-to-cell contact. ⌖ High bystander effect with MeP and F-Ade. ⌖ F-Ade is the toxic product of F-dAdo and F-araAMP. ⌖ Toxic metabolites diffuse freely even through the BBB.	⌖ Short plasma half-life. ⌖ Toxic effect shows schedule dependency. ⌖ Limited activation of F-adenine to toxic metabolites.	⌖ Trigger tumor cell death by DNA synthesis inhibition, block mRNA and protein synthesis.

*1 TK/GCV: Thymidine kinase/Ganciclovir, *2 CD/5FC: Cytosine deaminase/5-Fluorocytosine,

*3 CD/TK: Cytosine deaminase/Thymidine kinase,*4 PNP: Purine nucleoside phosphorylase

Table 5 Suicide gene therapy - Literature review and clinical trials.

Model	**Vector**	**Constructions**	**Pathway/ concentration/time with GCV**	**Results (tumour size /survival)**	**Notes accordingly to the construction used in the reference**	**Reference**
Murine	Retroviral	HSVTK[30] TKiresGFP IL4-TK	IP/IV 5-50 mg/kg/ day 1 - 2 weeks.	>50% tumor size reduction 100% TK full eradication	Mutant TK[30] has 6 aminoacid changes near the active site. It increases 35-fold the Km of thymidine kinase. Apoptosis is an important mechanism for cell death after HSV*tk*/GCV treatment. Tumor TK stimulates astroglia. Upregulation of heat shock proteins.	Gene therapy (1999) 6:1415-1426; Cancer Research (1999) 59 : 2384-2394; Cancer Gene Therapy (2000) 7:413-421; Cancer Gene Therapy (2000) 7:486-494
	Adenoviral	RSV-HSV*tk* wt p53 + HSV*tk*	IP 25-100 mg/kg/ day 1 - 2 weeks.	>70% survival over 3 months (ctrl 20 days)	HSV*tk* half-life 14–29 hours. Exogenous p53 enhance sensitivity to HSV*tk*/GCV.	Journal of Neuroscience Research (1994) 39 : 506 – 511; Nature Medicine (1999) 5:1256-1263; Molecular Therapy (2001) 4:490-498.
Human (Recurrent GBM Phase I/II/III study)	Retroviral /Adenoviral Intratumoral injection.	HSV*tk*	IV 10 mg/kg/day 2 weeks.	25% more than 1 year survival; <8% survival of 2,8 years.	From no side effects developed to headache, cerebral hematomas, thromboembolism, complications associated with the presence of catheter for GCV infusion, increased confusion, seizures, hyponatremia.	Human Gene Therapy (1998) 9:2595-2604; Endocrine Reviews (2004) 25:1-44; Molecular Therapy (2000) 1(2):195-203; Clinical Cancer Research (2002) 8(8): 2725–2734; Molecular Therapy (2004) 10(5):967-72.

III Problematic and goals

III.1 Problem

Lentiviral vectors are a promising tool for gene therapy, but they are a double-edged sword. They make stable and long lasting transgene expression possible by inserting their DNA into the chromosomes of transduced cells. At the same time, this chromosomal insertion is able to promote the expression of oncogenes or inactivation of tumor suppressor genes that eventually could give place to cancer development [82, 92].

III.2 General goal

Our general goal was to develop a safer lentiviral vector to produce antiangiogenic proteins to reduce glioma growth. On one hand, we wanted to be able to identify the cells transduced with this vector, to isolate them from mixed cultures and track them after transplantation *in vivo*. On the other hand, in the event of using such a vector in a clinical setting, we wanted to be able to kill the transduced cells on command in case of toxicity and oncogenicity. Therefore, we decided to use a bicistronic lentiviral construct expressing HSVtk-EGFP to render tracktable and ganciclovir sensible the lentiviral vector chosen to deliver antiangiogenic genes with the goal of examining their therapeutic potential.

III.3 Hypothesis

For the study with Angpt3, the general hypothesis was that this molecule would compete with Tie2 inactivation by Angpt2. While in the case of the soluble part of the receptor Tie2 (sTie2), it would sequester Angpt2. Therefore, both molecules should activate the Tie2 receptor intracellular signaling to favor the stability of tumor blood vessels and blocking GBM growth.

III.4 Specific aims

The first goal of this study was to improve the usability and safety of the lentiviral vector chosen to deliver potential therapeutic genes. On one hand, we wanted to be able to identify the cells transduced with this vector to isolate them from mixed cultures and track them after transplantation *in vivo*. On the other hand, in the event of using such a vector in a clinical setting, we wanted to be able to kill the transduced cells on command in case of problems. Indeed, although lentiviral vectors are promising tools for gene therapy, they pose toxicity and oncogenicity risks, and strategies need to be developed to increase their safety [80]. Therefore, we inserted into our lentiviral vector a bicistronic construct expressing a fusion protein composed of the marker enhanced green fluorescent protein (EGFP) and the suicide gene herpes simplex virus 1-thymidine kinase (HSV*tk*). Using such a vector, it is possible to kill cells expressing HSV*tk* by administration of ganciclovir, a nucleotide analogue that can be incorporated into DNA of proliferating cells once phosphorylated by HSV*tk*, which blocks DNA synthesis and induces apoptosis [93].

The second goal was to examine the therapeutic potential of two proteins that could presumably interfere with Angpt2, namely Angpt3 and the soluble extracellular domain of Tie2 (sTie2). The rationale was that Angpt3 would compete with Angpt2 for binding to Tie2 on the coopted vasculature, whereas sTie2 would sequester Angpt2. In support of this concept, it has been shown that administration of sTie2 inhibits the growth and metastasis of carcinoma and melanoma cells in mice by exerting antiangiogenic activity [64, 94, 95]. Similarly, it has been reported that overexpression of Angpt3 inhibits the ability of carcinomas to metastasize in the lungs [66], an organ in which Angpt3 induces Tie2 phosphorylation and activation [63].

Chapter 2: Methodology

2.1 DNA constructions

Two plasmids expressing HSVtk in fusion with EGFP were constructed (Figure 5a). Briefly, the cDNA encoding a mutant HSVtk (mutant 30 [96]; kindly provided by Dr. Margaret Black, Washington State University) was cloned into the pEGFP-C1 vector (BD Biosciences). The same cDNA, but without the stop codon, was cloned into the pEGFP-N1 vector (BD Biosciences). The resulting plasmids (pHSVtk-EGFP and pEGFP-HSVtk) were used for transfection.

Three lentiviral vectors expressing HSVtk-EGFP with or without Angpt3 or sTie2 were constructed (Figure 6a). First, the HSVtk-EGFP fusion gene was subcloned from the pHSVtk-EGFP plasmid into the multicloning site B of the pIRES plasmid (Invitrogen). Second, the Angpt3 and sTie2 cDNAs were amplified by RT-PCR using total RNA from mouse heart and the following primers: Angpt3, forward 5'-GGATAGGCTAGCATGCTC-TGCCAGCCAGCTATGC-3', reverse 5'-CTGCTTGAATTCTCAGGCACCCATTGGC-CTCAGC-3'; sTie2, forward 5'-ATAGGCTAGCATGGACTCTTTAGCCGGCTTAG-3', reverse 5'-GAATTCTCGAGTTATCCGAGGTCTGCAGAGGCTGGG-3'. The amplicons were separately cloned into the multicloning site A of the pIRES plasmid containing HSVtk-EGFP. Third, the portion of the resulting plasmids flanked by the CMV promoter and HSVtk-EGFP was PCR amplified (forward primer, 5'-TTTAAACGGCGCGC-CTCAATATTGGCCATTAGCC-3'; reverse primer, 5'-GGGAATTCGAATTACTTGTA-CAGCTCGTCCATG-3') and cloned between the cPPT and WPRE elements of a pHX-based vector [97]. The resulting constructs (pHR-Angpt3, pHR-sTie2, and pHR-Control) were sequenced to confirm identity and used for viral production and transduction.

2.2 Viral production

Each lentiviral vector was used to produce vesicular stomatitis virus G-pseudotyped lentiviruses by triple transfection as described previously [97]. Viral supernatants were concentrated by ultracentrifugation at 50 000 g for 90 min at 4°C. Viral titers were estimated by counting the number of EGFP+ 293T cells by flow cytometry two days after transduction with serial dilutions of the viral stocks.

2.3 Cell line and culture conditions

The murine glioma cell line GL261, originally propagated in B6 mice [98] and recently characterized [99], was provided by Dr. Protul Shrikant (Roswell Park Cancer Institute, Buffalo, New York). The cells were cultured in Dulbecco's modified Eagle's medium (Wisent, Saint-Bruno, Quebec, Canada) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 110 mg/L sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.4 Transfection

One day after seeding in T25 flasks, GL261 cells were transfected with 5 μg of linearized plasmid (pHSVtk-EGFP, pEGFP-HSVtk, or pEGFP-N1) using Lipofectamine and Plus Reagent according to the manufacturer's instructions (Invitrogen). The medium was replaced the following day with normal medium and then every 2-3 days with medium containing 400 μg/ml G418 antibiotic (Sigma-Aldrich). After two weeks, resistant cells were collected and sorted using an Epic Elite ESP flow cytometer (Beckman Coulter) to ensure purity and similar fluorescence intensity.

2.5 Transduction

One day after seeding, GL261 cells were incubated for 24 h in the presence of lentiviruses (pHR-Angpt3, pHR-sTie2, or pHR-Control) at a multiplicity of infection of ~50. Four days later, EGFP positive cells were sorted by flow cytometry. The cells were expanded and sorted twice more to ensure purity and similar fluorescence intensity.

2.6 Cell growth assays

After transfection or transduction (see above), GL261 cells were seeded in 6-well plates at a density of 20 000 cells per well. One day later, the medium was replaced with fresh medium supplemented or not with 0.1, 1 or 10 μM GCV (Hoffmann-La Roche; stock solution: 83 mg GCV, 20 ml saline, 15 μl HCl). Control cells were treated identically,

except that GCV was substituted with saline. At the indicated times, cells were collected by brief trypsinization and counted with an Epic XL flow cytometer (Coulter) by excluding dead cells by propidium iodide staining. In some experiments, to estimate the proportion of proliferating cells, cells were fixed for 30 min with 70% ethanol, stained for 30 min with a solution containing 50 µg/ml propidium iodide and 1.4 mg/ml RNase A, then analyzed by flow cytometry with Multicycle software (Phoenix Flow Systems). In addition, the percentage of viable cells (annexin V^-, 7-amino-actinomycin D^-) was estimated by flow cytometry using phycoerythrin-conjugated annexin V (Invitrogen) according to the manufacturer's protocol.

2.7 Western blotting

GL261 cells were seeded in T75 flasks. At confluence, the medium was replaced with VP-SFM (Invitrogen). Twenty-four hours later, the medium was collected, filtrated at 0.22 µm, supplemented with 1× protease inhibitor cocktail (Sigma-Aldrich), concentrated ~25 times with Centricon columns (10 000 molecular weight cutoff, Millipore), quantified for proteins by the Bradford method, and stored at –20°C in 2× loading buffer (160 mM Tris-HCl at pH 6.8, 4% SDS, 10% 2-β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue). For Western blotting, 20 µg of each sample were boiled for 2 min, separated on SDS-PAGE (5% stacking gel, 10% separating gel) and transferred to nitrocellulose membranes. The membranes were blocked for 16 h with 5% skimmed milk in TS buffer (10 mm Tris-HCl, 150 mm NaCl, pH 7.4), then incubated for 2 h with goat anti-Angpt3 (1 µg/ml; R&D Systems), anti-Tie2 (1 µg/ml, R&D Systems), or anti-AngptL4 antibody (2 µg/ml, R&D System) at room temperature. The membranes were washed and incubated for 1 h with peroxidase-conjugated anti-goat antibody (1:2500, Jackson Immunoresearch). After several washes, the membranes were incubated with an enhanced chemiluminescence system (Amersham) according to the manufacturer's instructions and exposed to Kodak Biomax light film. The biotinylated protein ladder detection pack (Cell Signaling Technology) was used to estimate protein size.

2.8 Intracerebral implantation of glioma cells

Two-month-old male C57BL/6 mice (Charles River Laboratories, Montreal, Quebec, Canada) were anesthetized, shaved and immobilized in a stereotaxic frame. A midline incision was made on the scalp, followed by a circular craniotomy over the right hemisphere, 1.7 mm lateral and 1 mm rostral from bregma. After removal of the dura mater, a 5-µl Hamilton syringe fitted with a 27-gauge beveled needle was advanced into the caudoputamen at a depth of 3.5 mm from the skull surface. Using a UMPII micropump (World Precision Instruments), 2 µl of Dulbecco's phosphate-buffered saline (Invitrogen) containing 5×10^4 viable GL261 cells were injected over 2 min. After injection, the syringe was left in place for 2 min before being withdrawn slowly. All procedures were performed in accordance with current guidelines of the Canadian Council on Animal Care.

2.9 GCV injection

Starting 3 days after glioma cell implantation, mice were injected intraperitoneally twice daily (~8 a.m. and 5 p.m.) for 14 or 60 days with 100 mg/kg of GCV (Hoffmann-La Roche) diluted in saline. Control mice were treated identically, except that GCV was substituted with saline.

2.10 Bromodeoxyuridine labeling

Bromodeoxyuridine (BrdU; Sigma-Aldrich) was dissolved in saline at a concentration of 10 mg/ml. Mice were injected intraperitoneally three times with BrdU (100 µg/g) at 2 h intervals and killed 2 h after the last injection.

2.11 Histological preparation

For all histological analyses except *in situ* hybridization, mice were transcardially perfused with 10 ml of saline, followed by ice-cold 4% paraformaldehyde in phosphate buffer, pH 7.4, over 10 min. The brains were removed, postfixed for 4 h at 4°C, then cryoprotected overnight in 50 mM potassium phosphate-buffered saline supplemented with 20% sucrose. Series of sections through the tumors were cut at 40 µm using a freezing microtome, collected in cryoprotectant (30% ethylene glycol, 20% glycerol, 50 mM sodium phosphate

buffer, pH 7.4) and stored at –20°C until analysis. For *in situ* hybridization, the following modifications were applied: 1) the fixative was dissolved in borate buffer, pH 9.5, instead of phosphate buffer; 2) the brains were postfixed for 48 h prior to overnight cryoprotection in the same fixative supplemented with 20% sucrose; and 3) sections were cut at 30 μm.

2.12 Immunostaining

Immunohistochemistry was performed as described previously [100] using the following primary antibodies: rat anti-BrdU (1:5000; Accurate Chemicals), rabbit anti-cleaved caspase-3 (1:500; Cell Signaling Technology), and rat anti-CD31 (1:500; BD Biosciences).

2.13 *In situ* hybridization

Brain sections were analyzed for Angpt3 or Tie2 mRNA by radioisotopic *in situ* hybridization as described previously [101]. Riboprobes probes were synthesized from linearized mouse cDNAs of ~1.5 kb in length.

2.14 Stereological analyses

For all the analyses described below, systematically sampled sections (every 10^{th} section) were examined in a blinded fashion using a Stereo Investigator system (Microbrightfield) combined with a Nikon E800 microscope.

Tumor volume was estimated from thionin-stained sections by the Cavalieri method. Using a 2× Plan Apochromat objective (numerical aperture 0.1), a point grid of 200 × 200 μm was overlaid on each section and the points that fell within the tumor were counted. Point counts were converted to volume estimates taking into account sampling frequency, magnification, grid size and section thickness.

Immunostained cells were counted by the optical fractionator method. Tumor tissue was traced using a 2× objective and sampled using a 60× Plan Apochromat oil objective (numerical aperture 1.4). The counting parameters were as follows: distance between counting frames, 400 × 400 μm ($BrdU^+$ cells) or 450 × 450 μm (cleaved caspase-3^+ cells); frame size, 30 × 30 μm ($BrdU^+$ cells) or 100 × 100 μm (cleaved caspase-3^+ cells); frame

thickness, 10 μm; guard zone thickness, ≥2 μm. Cells were counted if their body was at least partially within the 3D counting frame and did not touch the exclusion lines.

Tumor vessel density was evaluated from CD31-immunostained section by the area fraction fractionator method. Tumor tissue was traced using a 2× objective and sampled using a 40× Plan Apochromat oil objective (numerical aperture 0.95). The analysis parameters were as follows: counting frame size, 400 × 400 μm; distance between counting frames, 1000 × 1000 μm; grid size within counting frame, 35 × 35 μm. The number of points that fell on the vasculature and on the tumor in each counting frame was converted to area estimates taking into account sampling frequency, magnification and grid size.

Tumor vessel caliber was measured using the Quick Measure Line tool of Stereo Investigator. For unbiased sampling, a point grid of 325 × 325 μm was overlaid on each section and the vessel diameter was recorded systematically where points fell on the vasculature.

2.15 Survival analysis

After tumor implantation, mice were monitored daily and killed when any of the following criteria were observed: >20% weight loss, paralysis or lethargy. Survival time was calculated from the day of tumor implantation to the day of euthanasia or death. The Kaplan-Meier method was used to create survival curves, which were compared using the log-rank test.

2.16 Statistical analysis

Data are expressed as mean ± SE. Means were compared by one-way or two-way ANOVA, followed by the Tukey-Kramer test for multiple comparisons. Relationships between variables were assessed by Pearson correlation. All these analyses were performed with JMP software (SAS Institute) using an alpha level of 0.05.

Chapter 3: Results

3.1 Characterization of vectors expressing EGFP fused to HSVtk

To create a lentiviral vector capable of delivering a therapeutic gene while offering the possibility to identify and destroy transduced cells if desired, we first constructed plasmidic vectors expressing the marker EGFP fused to the C-terminal or N-terminal end of the suicide enzyme HSVtk under the control of the CMV promoter (Figure 10a).

To test whether the activity of the enzyme was preserved after fusion to EGFP, we transfected the plasmids into murine GL261 tumor cells, which are highly malignant cells of glial origin [99]. Stable transfectants were isolated and expanded before being exposed to different concentrations of the prodrug GCV for 3 days.

As determined by flow cytometry, GCV greatly reduced, in a dose-dependant manner, the number of cells transfected with either one of the plasmids expressing HSVtk fused to EGFP, but not the number of cells transfected with a control plasmid expressing EGFP alone (Figure 10b). Similar results were obtained after 2 days of treatment instead of 3 days (data not shown). We concluded that HSVtk was still active when fused to EGFP via its C-terminal or N-terminal end, so we arbitrarily selected the HSVtk-EGFP construct for future work.

We next constructed a lentiviral vector bearing a bicistronic cassette in which the HSVtk-EGFP gene was positioned as the second cistron (Figure 11a). Because the second cistron is usually less expressed than the first cistron, we initially sought to confirm the effectiveness of the vector in culture. Lentiviruses produced with the vector were used to transduce GL261 glioma cells, which were isolated, expanded, and exposed to different concentrations of GCV for 3 days. As shown in Figure 5c, a dose-dependent reduction in the number of transduced cells was observed, essentially confirming the results obtained with the plasmids in stable transfection (Figure 10b), although the effects were less pronounced. This was probably due to the lower expression of HSVtk-EGFP, as suggested by the mean fluorescence intensity of the cells, which was 4 (arbitrary units) compared to 40 for cells stably transfected with the pHSVtk-EGFP construct. Next, to evaluate the performance of the system *in vivo*, we implanted transduced GL261 cells into the brains of syngeneic mice. The cells were allowed to grow for 3 days and then the animals were

treated twice daily for 2 weeks with 100 mg/kg of GCV. Histological examination revealed that the tumors found in GCV-treated mice killed on the last day of treatment or 2 weeks later were ~37 times smaller than those found in saline-treated mice (Figure 10d-g). This regimen was not curative, because mice kept alive without additional treatment ultimately succumbed to gliomas, although they survived for a longer period (Figure 10h). To determine whether mice could be cured with a prolonged regimen, the experiment was repeated, except that mice were treated for 2 months instead of 2 weeks. As shown in Figure 10i, we found that 30% of the animals were tumor-free after more than 200 days. No noticeable side effect, such as a change in body weight, was associated with administration of GCV. Taken together, our results demonstrate that a bicistronic lentiviral vector expressing HSVtk-EGFP offers the possibility to identify and destroy transduced cells *in vivo*, even if they are aggressive tumor cells located behind the blood-brain barrier.

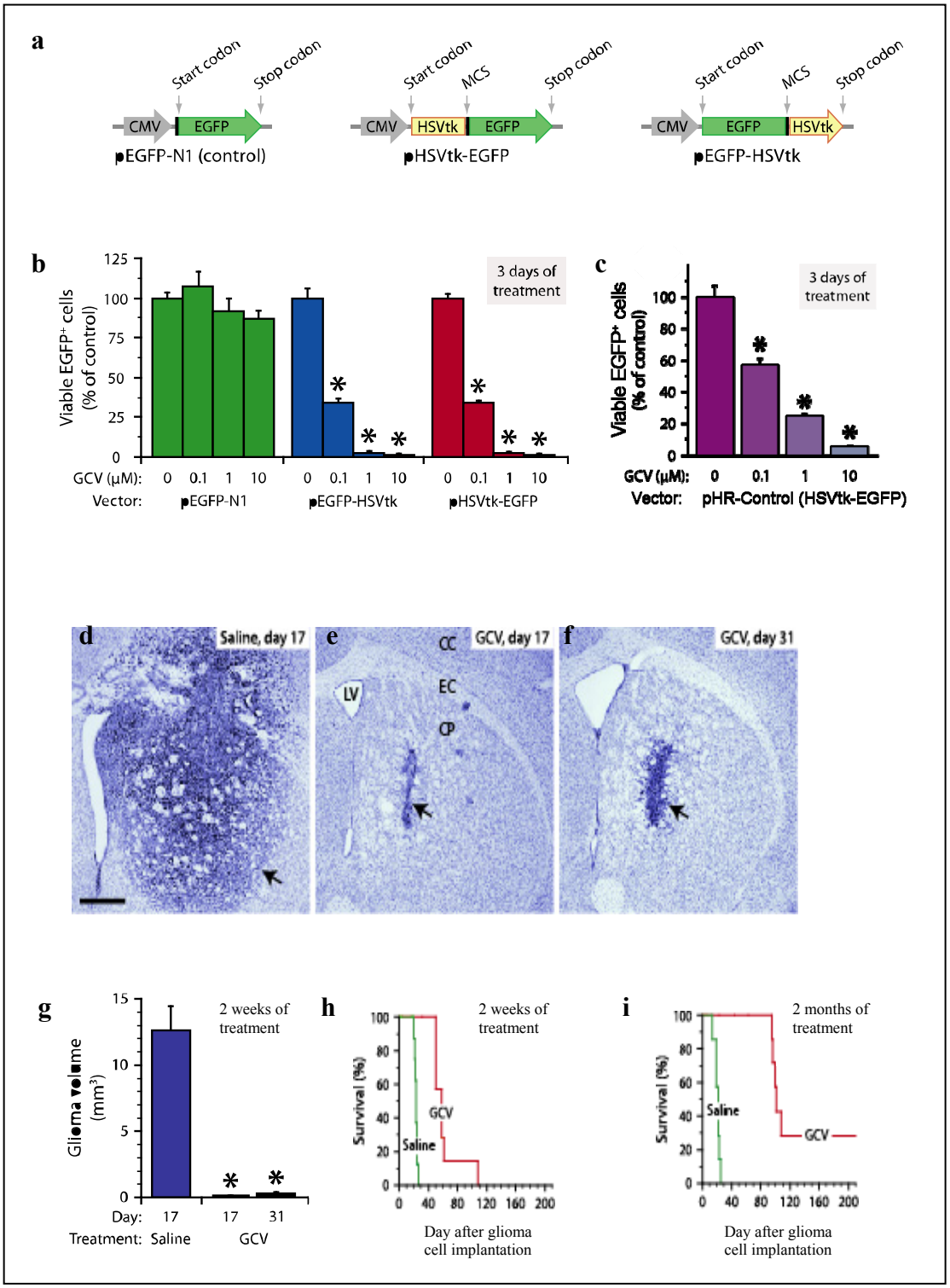


Figure 10 Characterization of vectors expressing HSVtk in fusion with EGFP.

Legend of figure 10. **a,** Diagram of the constructs used to express EGFP alone or fused to HSVtk. Abbreviations: CMV, cytomegalovirus promoter; MCS, multicloning site. **b,** As determined by flow cytometry, the number of viable GL261 glioma cells stably transfected with plasmids expressing EGFP-HSVtk or HSVtk-EGFP was reduced 3 days after treatment with different concentrations of GCV. No difference was observed between the pEGFP-HSVtk and pHSVtk-EGFP plasmids. *Significantly different from control (two-way ANOVA: treatment, $P = 0.0055$; vector, $P < 0.0001$; interaction, $P = 0.38$). $n = 3$ per group. **c,** The number of viable GL261 cells transduced with a pHR-based lentiviral vector expressing the HSVtk-EGFP construct was also reduced after GCV treatment. *Significantly different from control (ANOVA, $P < 0.0001$). $n = 3$ per group. **d-f,** Brain sections from mice injected intracerebrally with GL261 cells transduced with the pHR-Control (HSVtk-EGFP) lentiviral vector, and treated for 2 weeks with saline or GCV. The treatment started 3 days after glioma cell implantation, and the animals were killed either on the last day of treatment (day 17) or 2 weeks later (day 31). The images show gliomas (arrows) stained with thionin. Abbreviations: CC, cerebral cortex; CP, caudoputamen; EC, external capsule; LV, right lateral ventricle. Scale bar: 500 μm. **g,** Stereological analysis revealed a 37-fold reduction in glioma volume in mice treated with GCV. *Significantly different from saline-treated mice (ANOVA, $P < 0.0001$). $n = 7$-8 per group. **h, i,** Kaplan-Meier curves showing an increase in survival after treatment with GCV for 2 weeks or 2 months, respectively. Log-rank test, $P < 0.0001$. $n = 7$-8 per group.

3.2 Transgenic expression of Angpt3 and sTie2 in glioma cells

To study the effect of Angpt3 and sTie2 on glioma growth, we constructed two bicistronic lentiviral vectors expressing either of these proteins by positioning the corresponding cDNAs as first cistrons (Figure 11a). The parental vector expressing only HSVtk-EGFP was used as a control. Lentiviruses produced with these three vectors were used to transduce GL261 cells in culture, which were isolated by FACS on the basis of EGFP fluorescence (Figure 11b). Transgenic expression of Angpt3 and sTie2 was confirmed *in vitro* by Western blotting using culture supernatants, as well as *in vivo* by *in situ* hybridization using brain sections from mice killed 2 weeks after implantation of transduced cells (Figure 11c, d).

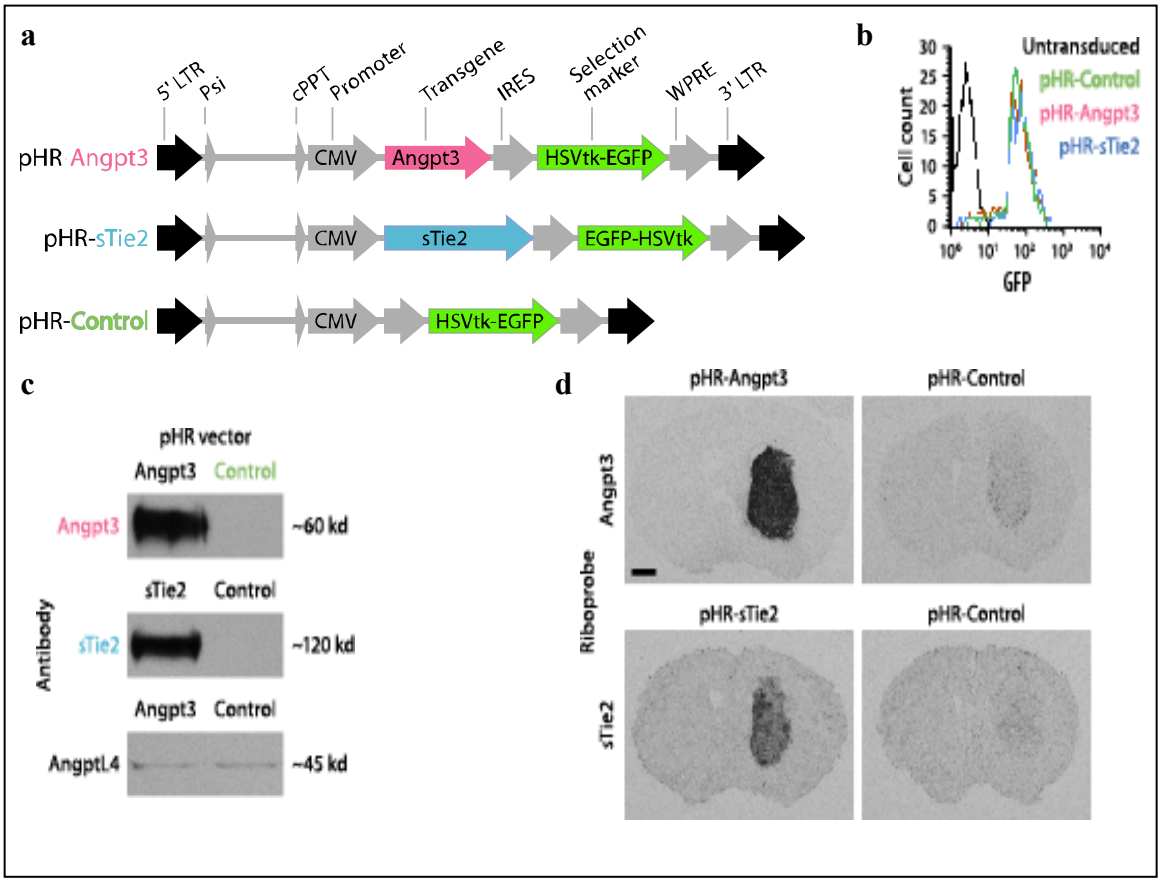


Figure 11 Characterization of bicistronic lentiviral vectors coexpressing Angpt3 or sTie2 with HSVtk-EGFP.

Legend of figure 11. **a,** Diagram of the vectors. Abbreviations: CMV, cytomegalovirus promoter; cPPT, central polypurine tract; IRES, internal ribosome entry site; LTR, long terminal repeat; Psi, packaging signal; WPRE, woodchuck hepatitis post-transcriptional regulatory element. **b,** Flow cytometric analysis of GL261 cells transduced with the vectors and purified by FACS. Comparable levels of EGFP expression were observed among the transduced cells. **c,** Western blots showing Angpt3 or sTie2 in culture supernatant from GL261 cells transduced with the corresponding vector. An antibody against angiopoietin-like 4 (AngptL4) was used as a control for protein loading. **d,** *In situ* hybridization for Angpt3 or sTie2 mRNA in brain sections from mice killed 2 weeks after implantation of GL261 cells transduced with the corresponding vector. Scale bar: 1 mm.

3.3 No effect of Angpt3 and sTie2 on glioma cell growth *in vitro*

Because Tie2 expression is generally restricted to endothelial cells [70], we expected no direct effect of Angpt3 or sTie2 on GL261 cells. In agreement with this, we found that GL261 cells transduced with the Angpt3, sTie2 or control vector grew at a similar rate in culture (Figure 12a). In addition, there was no intergroup difference in the percentage of cells in the S, G2, and M phases of the cell cycle (Figure 12b), as well as in the percentage of apoptotic cells (Figure 12c). Overall, these results confirm that Angpt3 and sTie2 do not directly affect the proliferation and survival of GL261 cells.

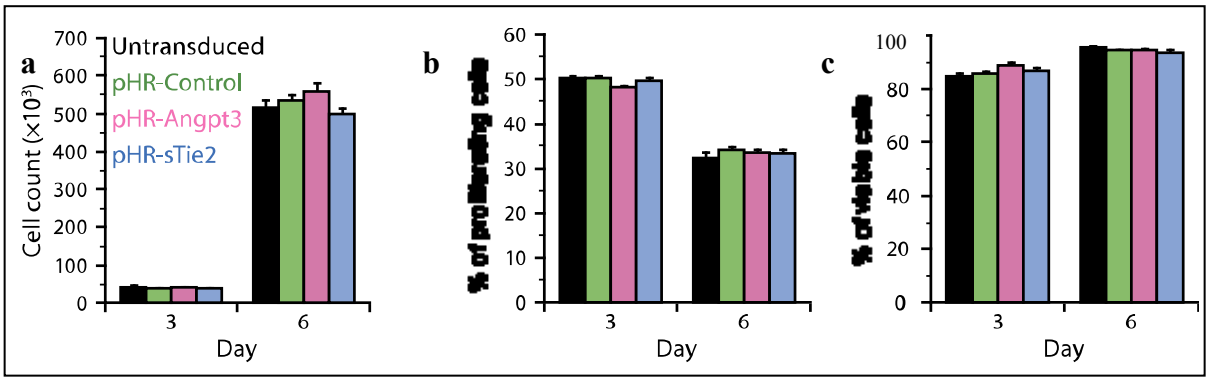


Figure 12 Proliferation and viability of GL261 cells expressing Angpt3 or sTie2.

Legend of figure 12. **a,** As determined by flow cytometry, the total number of cells generated in culture was similar in the case of GL261 cells untransduced (wild-type cells, black bar) or transduced with a lentiviral vector expressing Angpt3 (red bar), sTie2 (blue bar), or only HSVtk-EGFP (control vector, green bar). Two-way ANOVA: treatment effect, $P > 0.05$. $n = 6$ per group. **b, c,** No intergroup difference was observed in cell proliferation (*i.e.*, the proportion of cells in the S and G2/M phases of the cell cycle) and cell viability (*i.e.*, the proportion of cells double negative for annexin V and 7-amino-actinomycin.

3.4 Influence of Angpt3 and sTie2 on orthotopic glioma growth

To assess the effect of Angpt3 and sTie2 on glioma growth *in vivo*, we implanted the transduced cell lines into the brains of syngeneic mice, which were killed 21 days later for histological analysis. Before sacrifice, the animals were injected with BrdU to label dividing cells. Unbiased volumetric analysis revealed that Angpt3-expressing gliomas were in average 62% larger than control gliomas, whereas no difference was observed in the case of sTie2-expressing gliomas (Figure 13a, b). Furthermore, we did not detect intergroup difference in the number of $BrdU^+$ cells and cleaved caspase-3^+ cells within the tumors (Figure 13c-f). Altogether, these results indicate that transgenic expression of Angpt3 promotes glioma growth and that this effect is not due to a change in the rate of proliferation or apoptosis.

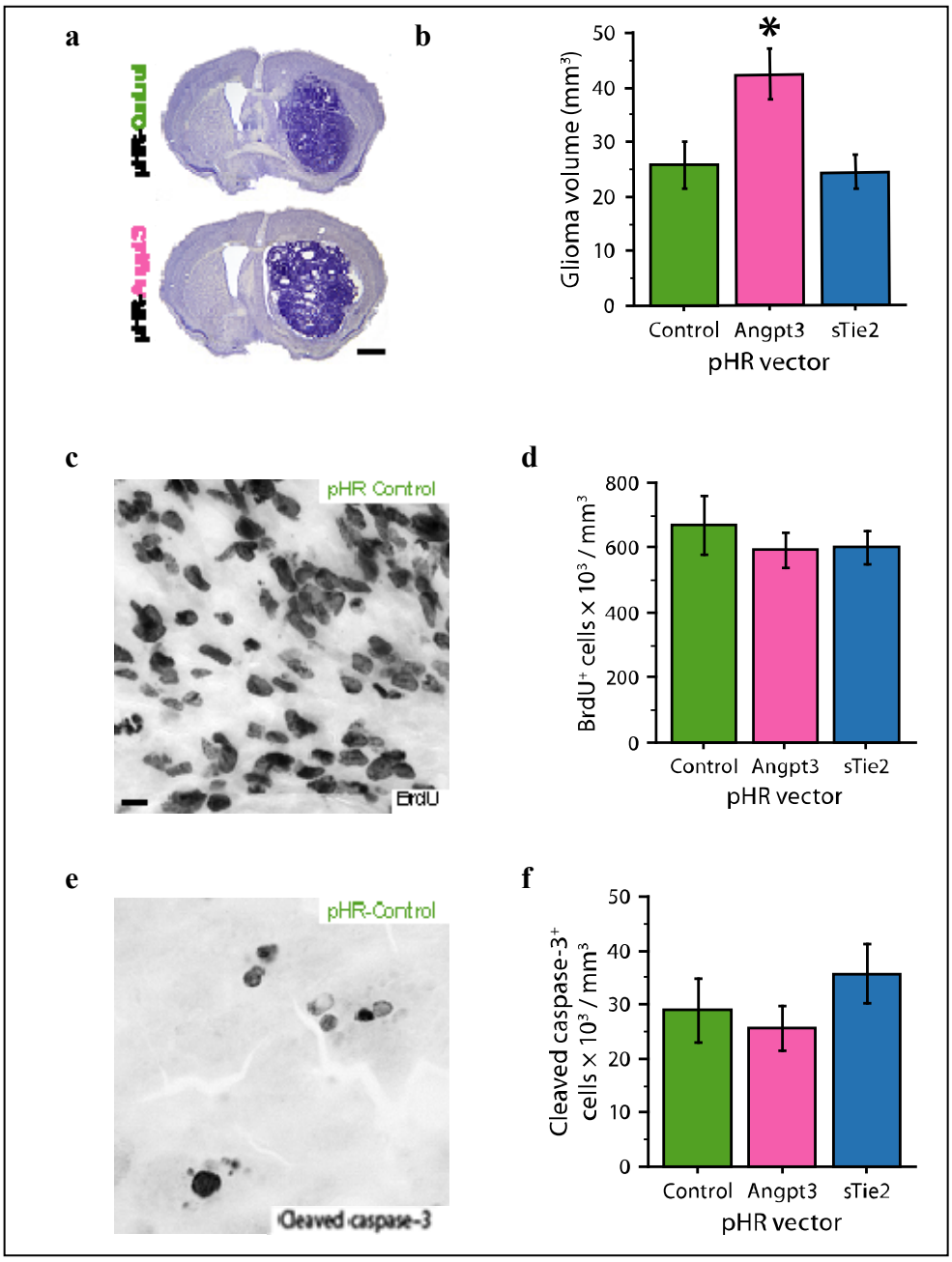


Figure 13 Influence of Angpt3 and sTie2 on glioma growth.

Legend of figure 13. **a,** Representative glioma sections stained with thionin from mice killed 21 days after implantation of GL261 cells into the right caudoputamen. Scale bar: 1 mm. **b,** Stereological analysis revealed that gliomas expressing Angpt3 were 62% larger than control gliomas. *Statistically different from the other groups (ANOVA, $P = 0.0077$). n = 9-11 per group. **c,** Proliferating cells in a control glioma stained for BrdU by immunohistochemistry. Scale bar (*c, e*): 10 μm. **d,** No intergroup difference was detected in cell proliferation (ANOVA, $P = 0.68$). $n = 5$ per group. **e,** Apoptotic cells in a control glioma stained for cleaved caspase-3 by immunohistochemistry. **f,** No intergroup difference was detected in cell apoptosis (ANOVA, $P = 0.38$). n = 9-11 per group.

3.5 Influence of Angpt3 and sTie2 on glioma vasculature

To examine whether Angpt3 and sTie2 affected the vasculature, we immunostained glioma sections for the endothelial marker CD31. Stereological analyses revealed a 38% increase in vessel diameter in Angpt3-expressing gliomas compared to control gliomas, but no difference in the case of sTie2-expressing gliomas (Figure 14a-c). Interestingly, we found a strong positive correlation between vessel diameter and glioma volume (Figure 14d). Although we also found a correlation, while modest, between vessel density and glioma volume, no intergroup difference was detected in vessel density (Figure 14e, f). Therefore, contrary to our initial hypothesis, these results suggest that Angpt3 can destabilize the tumor vasculature as reported for Angpt2 [70].

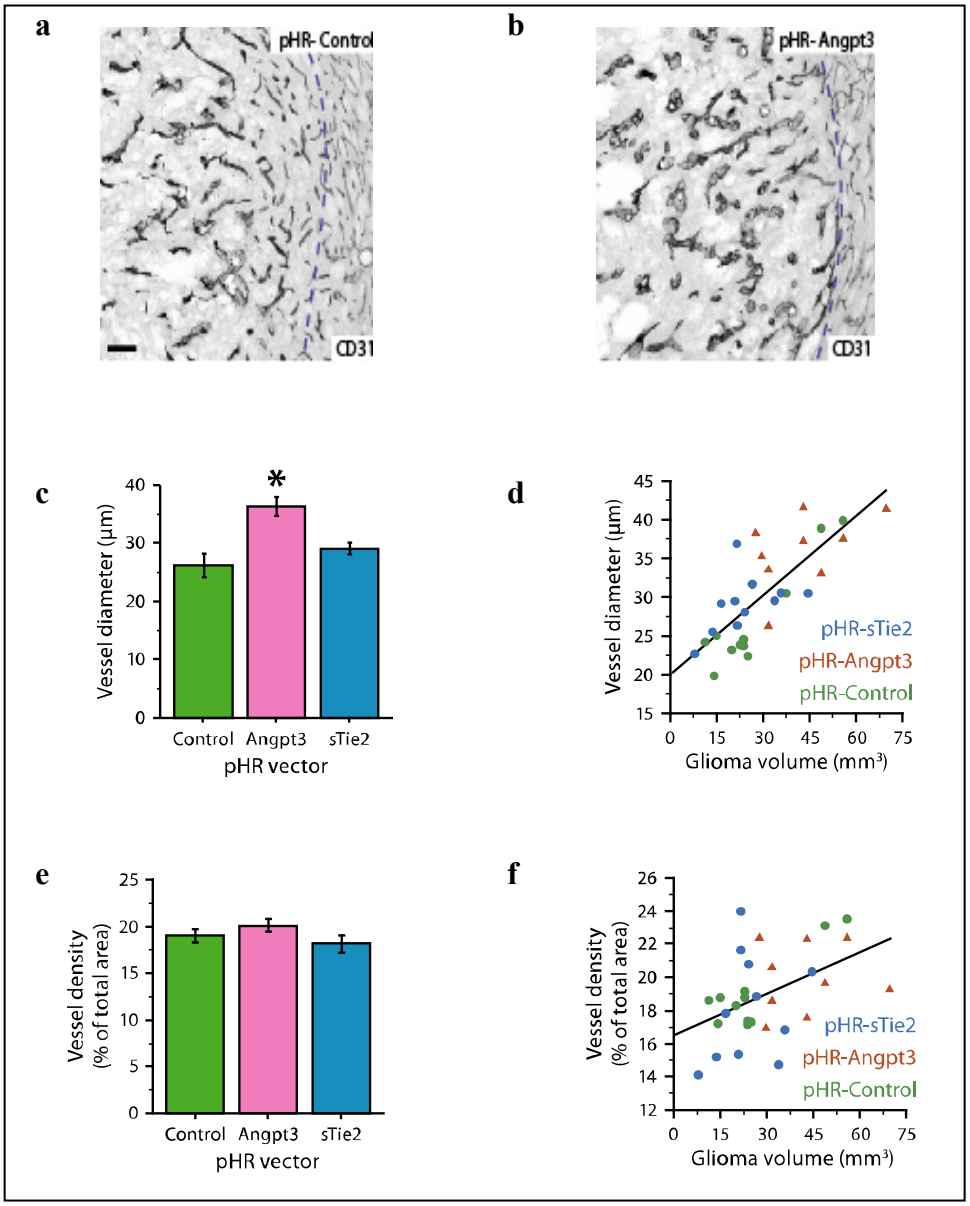


Figure 14 Influence of Angpt3 and sTie2 on glioma vascularization.

Legend of figure 14. **a, b,** Glioma sections stained for CD31 by immunohistochemistry. The dashed line separates the tumor (left) from the normal tissue (right). Scale bar (*a, b*): 10 mm. **c,** Stereological analysis revealed a 38% increase in vessel diameter in gliomas expressing Angpt3 compared to control gliomas. *Statistically different from the other groups (ANOVA, $P = 0.0007$). $n = 9\text{-}11$ per group. **d,** A strong correlation was found between glioma volume and vessel diameter (Pearson correlation, $P < 0.0001$, $R = 0.77$). **e,** No intergroup difference was detected in vessel density (ANOVA, $P = 0.27$). $n = 9\text{-}11$ per group. **f,** A correlation was found between glioma volume and vessel density (Pearson correlation, $P = 0.0074$, $R = 0.47$).

Chapter 4: Discussion

Malignant brain tumors alter the structure and function of blood vessels, thereby promoting their growth and edema formation. This later complication can be managed by the use of dexamethasone, a synthetic glucocorticoid that can normalize the vasculature, but only partially and with potential side effects (*e.g.*, cushingoid symptoms, opportunistic infections)[101]. With the long-term goal of identifying a more effective and safer treatment for brain cancer, we studied two molecules previously reported as being antiangiogenic. Contrary to our expectations, we found that sTie2 exerts no detectable effect in a glioma model, whereas Angpt3 increases blood vessel destabilization and glioma growth. Although this study does not support the idea of using these molecules to treat brain cancer, it helps to better understand the function of Angpt3 and the importance of the Tie2 signaling pathway in glioma development. This study also offered us the opportunity to develop a more useful and safer expression cassette that can be used for experimental or therapeutic purposes.

Lentiviral vectors are powerful tools for gene transfer, but their use in human raises biosafety concerns. For example, they can potentially cause cancer by insertional mutagenesis or by carrying a transgene that provides cells with a growth advantage [80]. In addition, as they permanently integrate into the host genome, the transgene could become toxic if transduced cells proliferate and disseminate to other organs in an unwanted manner. Our lentiviral bicistronic construct expressing HSVtk-EGFP should help to attenuate these concerns by allowing the tracking and killing of transduced cells, even if they are located behind the blood-brain barrier. Indeed, we showed that it is possible to stop the growth of transduced glioma cells implanted into mouse brains as long as the animals are treated with GCV. These cells can even be totally eradicated with an appropriate regimen of GCV. Although the effectiveness of the vector was satisfactory, it could be advantageous in the future to further improve it to increase fluorescence intensity and cell sensitivity to GCV. For example, the IRES sequence could be optimized to enhance the expression of the HSVtk-EGFP cistron, while EGFP could be replaced with a brighter marker (*e.g.*, tdTomato).

An important question that arises from our work is how Angpt3 increased glioma growth. The answer is probably not by affecting glioma cell proliferation or survival, since we found no evidence of this *in vitro* and *in vivo*, although we can not exclude subtle spatiotemporal changes undetectable by the methods used. Because Tie2 is mostly restricted to endothelial cells [50], we propose instead that Angpt3 acted by destabilizing the cerebral endothelium, as previously observed in the case of Angpt2 [70, 102]. This effect would be restricted to vessels in direct contact with glioma cells, because Angpt3 is tethered on the cell surface via heparan sulfate proteoglycans [103]. Although it has been shown that Angpt3 acts as an agonist of Tie2 on the endothelium of the lung, an organ in which it is constitutively produced [63], it is possible that Angpt3 blocks Tie2 signaling at ectopic sites like the brain. Such context-specific effects have been observed for Angpt2 and could be attributable to the presence or absence of accessory molecules involved in the angiopoietin–Tie2 signaling pathway. This could explain why Angpt3 overexpression inhibits pulmonary metastasis [66], but not glioma growth.

It is widely accepted that blocking Tie2 signaling destabilizes the endothelium [29, 50, 67], but how this destabilization would increase glioma growth is unclear. One hypothesis is that the edema resulting from breakdown of the blood-brain barrier promotes glioma growth by contributing to the destruction of the peritumor tissue. This hypothesis derives from the property of malignant gliomas to infiltrate the surrounding tissue by causing its destruction [104]. Indeed, it has been reported that glioma cells produce toxic levels of glutamate that facilitate their progression, an effect that can be blocked by the administration of the glutamate receptor antagonist MK801 [105] or the glutamate transporter inhibitor S-4-carboxyphenylglycine [106]. Similarly, other potentially toxic molecules derived from plasma that penetrates the parenchyma through a damaged blood-brain barrier could promote glioma cell invasion. We therefore propose that Angpt3 exerted a protumor effect in our glioma model by destabilizing the cerebral endothelium, thereby causing alterations in the peritumor tissue that facilitated the progression of glioma cells. Further investigation will be required to validate and characterize this mechanism.

It has been shown that sTie2 can reduce the growth of subcutaneous tumors and lung metastases in mice by exerting antiangiogenic activity when injected directly into the tumors or delivered in the blood using an adenoviral vector [64, 94, 95]; however, sTie2 did not produce any effect in our glioma model. This discrepancy could be explained by fundamental differences in the models used. First, the peripheral tumor models could be more sensitive to sTie2, since they are critically dependent on Angpt2 for the formation of new blood vessels, contrary to our glioma model that grows without angiogenesis and is only modestly influenced by Angpt2 [70]. Second, sTie2 possibly exerts its effect preferentially on the luminal surface of the endothelium, where Angpt2 is released from the Weibel-Palade bodies [107]. Access of sTie2 to this compartment is possible when administered into the blood or an organ with fenestrated capillaries, but could be restricted in the brain where the capillaries have tight junctions. Whether sTie2 could reduce glioma growth when injected into the circulation remains to be determined, although this approach may not be advantageous over the use of anti-Angpt2 inhibitors such as L1-10 [70].

Chapter 5: Conclusion

In conclusion, this study warns that antiangiogenic treatments shown to be effective against certain cancers can be ineffective or even harmful in other cancers such as malignant gliomas. Nevertheless, together with previous work in the laboratory [70], this study supports the view that the Tie2 signaling pathway influences glioma development and represents an attractive therapeutic target. Finally, the bicistronic expression cassette developed in this study could be a new tool in our therapeutic arsenal that could help to make lentiviral gene therapy safer and ethically more acceptable. In experimental studies, this cassette could be used to eliminate the transduced cells, thereby providing an internal control.

To increase cell sensitivity to GCV, we could use the fusion of HSVtk with CD in order to synergize the effect and to be able to kill proliferating and non-proliferating cells. We could deplete dTTP in order to increase GCV uptake and, hence, the cytotoxic effect, as previously suggested by other authors (Table 5 and references therein). Alternatively, other possible improvements of the lentiviral vector developed in this study could be done at the level of the proteins of the viral envelope. Lately it has been shown that LV pseudotyped with glycoproteins of the Lymphocytic choriomeningitis virus (LCMV-GP) mediated efficient and specific transduction of rat glioma cells when compared with normal brain cells like astrocytes and neurons [108].

Endothelial cells in the tumor vasculature have a lower mutation rate compared with tumor cells, which means that endothelial cells are unlikely to acquire resistance to the therapeutic drugs. Angiogenesis is infrequent in the adult and therapies that tract angiogenic endothelial cells may have minimal toxicities. Tumor vascular targeting therapy as well as antiangiogenesis therapy could control tumor growth independently of tumor-cell type. Endothelial cells are easily accessible by intravenously administered therapeutic agents thus circumventing the problem of transcytosis across the vascular wall and diffusion through out tumor tissue. Additionally, vascular targeting combined with appropriate chemotherapy is more effective than either therapy alone [109].

One perspective is to apply multifactorial therapies to combat this disease. Transgene regulation will be crucial for maintaining appropriate levels of a gene product within the therapeutic range, thus preventing toxicity. Moreover, the possibility to modulate, stop or resume transgene expression in response to disease evolution would facilitate the combination of gene therapy with more conventional therapeutic modalities [110].

After suicide gene therapy

Cytotoxic methods non involving suicide gene therapy involves immunotherapy, transporter or channel modifications, metabolism signal, etc. One effective therapy for cancer has recently been described: *in vivo* expression of the $ASIC2^{G430F}$ mutant sodium channel inside cancer cells. In this study, the sodium channel is constitutively open allowing the unrestricted and lethal flow of sodium into cells expressing the channel and neighboring cells by bystander effect. The advantages of this system versus TK/GCV system is that it is able to kill non-dividing cells and it provides a robust killing mechanism for tumor cells by a rapid, explosive mechanism (>80% cell death as soon as 24 h post-infection). Safety features of this system include drug-inducible regulation of the transgene and a limiting effect through dilution of ions/water as they spread among cells through gap junctions, combined with delivery by intratumoral injection of the vector [111]. Other systems available for controlling the transgene expression are tetracycline-doxycicline, G419-hygromycin, zinc fingered artificial nucleases, etc.

Annexes

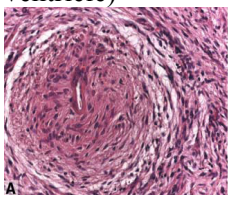
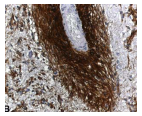
ANNEX I. WHO Classification of brain tumors 2007 [2].

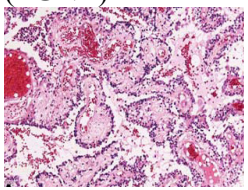
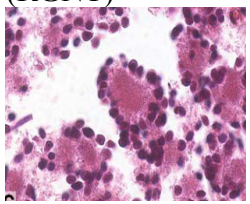
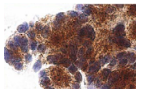
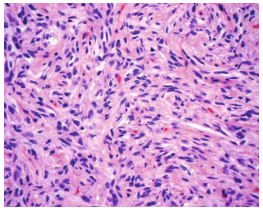
Astrocytic tumors	I	II	III	IV
Subependymal giant cell astrocytoma	*			
Pilocytic astrocytoma	*			
Pilomyxoid asrocytoma		*		
Diffuse astrocytoma		*		
Pleomorphic xanthoastrocytoma		*		
Anaplastic astrocytoma			*	
Glioblastoma				*
Giant cell glioblastoma				*
Gliosarcoma				*
Oligodendroglial tumors				
Oligodendroglioma		*		
Anaplastic oligodendroglioma			*	
Oligoastrocytic tumors				
Oligoastrocytoma		*		
Anaplastic oligoastrocytoma			*	
Ependymal tumors				
Subpendymoma	*			
Myxopapillary ependymomas	*			
Ependymoma		*		
Anaplastic ependymomas			*	
Choroid plexus tumors				
Choroid plexus papilloma	*			
Atypical choroid plexus papilloma		*		
Choroid plexus carcinoma			*	
Other neuroepithelial tumours				
Angiocentric glioma	*			
Choroid glioma of the 3rd ventricle		*		

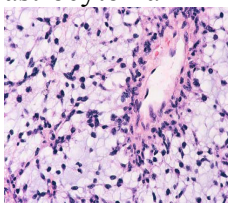
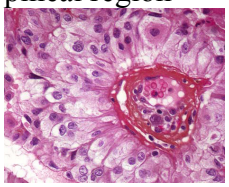
	I	II	III	IV
Central neurocytoma		*		
Extraventricular neurocytma		*		
Cerebellar liponeurocytoma		*		
Paraganglioma of the spinal cord	*			
Papillary glioneuronal tumor	*			
Rossette-forming glioneuronal tumor of the fourth ventricle	*			
Pineal tumors				
Pineocytoma	*			
Pineal parenchymal tumor of intermediate differentiation		*	*	
Pineoblastoma				*
Papillary tumor of the pineal region		*	*	
Embryonal tumors				
Medulloblastoma				*
CNS primitive neuroectodermal tumour				*
Atypical teratoid / rhabdoid tumor				*
Tumors of the cranial and paraspinal nerves				
Schwannoma	*			
Neurofibroma	*			
Perineurioma	*	*	*	
Malignant peripheral nerve sheath tumor		*	*	*
Meningeal tumors				
Meningioma	*			
Atypical meningioma		*		
Anaplastic / malignant meningioma			*	
Haemangiopericytoma		*		
Anaplastic haemangiopericytoma			*	
Haemangioblastoma	*			

Neuronal and mixed neuronal-glial tumors						Tumors of the sellar region				
Gangliocytoma	*					Craniopharyngioma	*			
Ganglioglioma	*					Granular cell tumor of the neurohypophysis	*			
Anaplastic ganglioglioma			*			Pituicytoma	*			
Desmoplastic infantile astrocytoma and ganglioglioma	*					Spindle cell oncocytoma of the adenohypophysis	*			
Dysembryoplastic neuroepithelial tumor	*									

Topography of brain tumors by grade of malignancy and specific glioma immunohistopathological characteristics [2]

Brain tumor type		**Grade WHO / ICD-O**	**Patient's mean age at surgery**	**Clinical symptoms & tumor behavior**	**Topography of the tumor**	**Microscopic features / Histopathology**	**Antigens / Markers**
Angiocentric glioma (astroblastoma and choroid glioma of the 3rd ventricle) 		I	17 years old	Refractory epilepsy Benign clinical behavior (Tumors are stable or slowly growing)	Located superficially Fronto- parietal cortex, temporal lobe and hippocampal region	Well delineated, hyperintense, non-enhancing cortical lesions often with a stalk like extension to the subjacent ventricle Monomorphous big polar cells, an angiocentric growth pattern and immunoreactivity	EMA GFAP  S-100 Vimentin No neuronal antigens
Pilocytic astrocytoma		I	0-20 years old	Benign clinical behavior. > 85% of patients survive 5 years	Cerebellum, optic nerve		NF1
Choroid Plexus	C.P. papilloma	I 9390/0		Benign clinical behavior			
	Atypical C.P.P.	II 9390/1		Higher probability of recurrence		Increased mitotic activity	
	C.P. Carcinoma	III 9390/3		Malignant (Tumor highly invasive)		Frank signs of malignancy (brisk mitotic activity, increased cellularity, necrosis and invasion of brain parenchyma	

Brain tumor type	Grade WHO / ICD-O	Patient's mean age at surgery	Clinical symptoms & tumor behaviour	Topography of the tumor	Microscopic features / Histopathology	Markers
Papillary glioneuronal tumor (PGNT) 	I 9509/1	Wide age range Mean 27	Benign neoplasm	Temporal lobe	Contrast-enhancing, well delineated mass showing a cyst-mural nodule pattern Single or pseudostratified layer of flat to cuboidal GFAP-positive astrocytes surrounding hyalinized vascular pseudopapillae	GFAP Synaptophysin Neuron/ Gangliod cells
Rosette-forming glioneuronal tumor of the 4th ventricle (RGNT) 	I 9509/1	Young adults mean age 33 years old	Slowly growing tumor of the 4th ventricle	4th ventricle; arise in the midline and primary involves the cerebellum	Well delineated, hyperintense tumor, perivascular pseudorosettes Biphasic neurocytic and glial architecture (similar to pilocytic astrocytoma)	Neurocytes + cells Eosinophilic + Synaptophysin + cores 
Pituicytoma 	I 9432/1	Adults	Visual disturbance, headache and features of hypopituitarism benign (slow growth)	Neurohypophysis or infundibulum 'posterior pituitary astrocytoma'	Solid masses well-circumscribed; can measure up to several centimeters. Elongate, bipolar spindle cells arranged in interlacing fascicle or assuming a storiform pattern. Mitotic figures are absent or rare.	VimentinS-100 GFAP

Brain tumor type		Grade WHO / ICD-O	Patient's mean age at surgery	Clinical symptoms & tumor behaviour	Topography of the tumor	Microscopic features / Histopathology	Markers
Neurocytoma	Central Extraventricular	II 9506/1	Young adults	Benign	Lateral ventricles in the region of the foramen of Monro	Composed of uniform round cells with low proliferation rate	Neuronal differentiation Mimicks neuropil
Pilomyxoid astrocytoma 		II 9425/3	Infants and children (median age, 10 months)	Local recurrences and cerebrospinal spread are more likely to occur in pilomyxoid than in pilocytic astrocytomas Prognosis less favorable	Hypothalamic / chiasmatic region	Myxoid matrix and angiocentric arrangement of monomorphous, bipolar tumor cells	
Ependymoma		II	Childhood and young adults	Less than 30% survive 5 years	Ventricles and spinal cord		NF1
Papillary tumor of the pineal region 		II/III 9395/3	Children and adults (mean age 32 years)	Malign	Pineal region	Neuroepithelial tumour relatively large (2.5–4 cm) well-circumscribed and showing contrast enhancement. Papillary architecture and epithelial cytology	Cytokeratin GFAP

Brain tumor type	**Grade WHO / ICD-O**	**Patient's mean age at surgery**	**Clinical symptoms & tumor behaviour**	**Topography of the tumor**	**Microscopic features / Histopathology**	**Markers**
Oligodendroglioma	II/III	Young and old adults	50% of patients survive 5 years	Cerebral hemispheres		LOH 1p and 19q
Anaplastic medulloblastoma	IV 9474/3				Marked nuclear pleomorphism, cell–cell wrapping, and high mitotic activity, often with atypical forms spherical cells with round nuclei, open chromatin and prominent central nucleoli	
Glioblastoma	IV	More than 45 years old	Malign clinical behavior Only 3% survive 5 years	Cerebral hemispheres		EGFR PTEN LOH chr10
Neuroblastoma	IV	Childhood (less than 20 years)	50% survive 5 years when older than 1 year. 90% survive longer than 5 years when younger than 1 year at diagnosis	Abdomen		MYCN Trisomy 17q LOH 1p, 11q

ANNEX II. Vectors used for gene therapy for central nervous system. Modified from [14, 112]

VECTOR	ADVANTAGES	DISADVANTAGES
Enveloped		
Retrovirus (RNA virus)	Target only dividing cells Large packaging capacity (7-10 Kb) Delivery in VPC* Not inactivated in CSF** Persistent gene transfer	Target only dividing cells Inactivated in plasma Insertional mutagenesis Replication competent retrovirus (RCR) Low titre and requires VPC*
Lentivirus (LV) (RNA virus)	Target dividing and non dividing cells Large packaging capacity (7-10 kb) Stable transgene expression Low antivector immunity	Insertional mutagenesis Revertant replication competent virus Low titers production
Herpes simplex virus (HSV) (DNA virus)	Neurotropism Large packaging capacity (40-150 kb) No DNA insertion in host genome (episomal) Short transgene expression High titres (replication-compromised)	Neurotoxicity Lytic infection Recombination to wild type of lytic virus Low titer (replication-defective)
Non-enveloped		
Adenovirus (AdV) (DNA virus)	Target non-dividing cells (episomal) Large capacity (7-10 Kb) Efficient transduction in most tissues Non-oncogenecity Low level of neurotoxicity	Target only non-dividing cells Host immune reaction Risk of replication Transient gene expression
Adeno-associated virus (AAV) (DNA virus)	Target non-dividing cells Less likely to produce immune reaction Persistent delivery capacity	Target only non dividing cells Risk of replication Small capacity (5 Kb)
Non-viral		
Liposomes (Non-viral)	Easy to produce Non-infectious Non-immunogenic	Transient gene expression No cell-type specificity Low levels of transgene expression

*VPC: Viral producer cell lines; **CSF: Cerebrospinal fluid

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