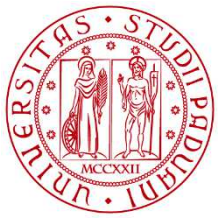

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SCUOLA DI DOTTORATO DI RICERCA IN BIOMEDICINA

INDIRIZZO COMUNE

CICLO XXVII

ENGINEERED BOVINE HERPESVIRUS TYPE 4: A NEW TOOL AGAINST GLIOMA STEM-LIKE CELLS?

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A chi ha sempre creduto in me,

anche quando io avevo smesso di farlo.

SOMMARIO

1. ABSTRACT

Glioblastoma (GBM) is a grade IV primary brain tumor (WHO 2007). Despite last progresses in surgery, chemo- and radiotherapy, this brain tumor is still incurable and it always recurs (Stupp *et al,* 2009). It has been hypothesized that its resistance to therapies and its recurrence might be due to the existence of a subpopulation of tumor cells called Glioma Stem Cells (GSCs)(Galli *et al,* 2004; Bao *et al,* 2006). The goal of GBM therapy is the selective targeting of GSCs and, among the possibilities, the use of viral vectors has been widely investigated (Kroeger *et al,* 2010). Herpesviruses have been largely studied and modified in GBM treatment (Grandi *et al,* 2009). In this study the capacity of an engineered Bovine Herpesvirus type 4 (BoHV-4), named BoHV-4TKdsRED, to selectively kill GSCs has been investigated. The vector expresses the HSV-1 Thymidine Kinase and so it can be used with Ganciclovir (GCV) in a Suicide Gene Therapy protocol. Its potential role as a vector in GBM treatment has already been demonstrated (Redaelli *et al,* 2012).

Three different primary cultures of human GSCs (GBM2, GBM4 and GBM5) and the corresponding serum-cultured cells (FBS2, FBS4 and FBS5) were used. They have been infected with BoHV-4TKdsRED and a combined treatment of the vector with GCV has also been tested on GSCs. It has also been analyzed whether signaling pathways of apoptosis or autophagy are activated in infected GSCs. To verify the BoHV-4TKdsRED effect on the cells counting/killing assays, plaque assays, Western Blot analysis and FACS analysis have been done.

BoHV-4TKdsRED is able to infect GSCs and FBS cells. It can slightly replicate in almost all cultures examined and it can kill them *in vitro*. The combined treatment of the vector with GCV leads to an increased mortality in GSCs. Initial signs of autophagy activation were detected with the combined treatment in GSCs.

Study of apoptosis through FACS analysis led to unreliable results.

Another BoHV-4-derived vector, BoHV-4EGFPΔTK, demonstrated its ability in infecting self-renewing cells, but without exerting a cytopathic effect.

According to our results, BoHV-4TKdsRED would be a promising vector to target GSCs, but further analysis should be performed to strengthen these data.

1. SOMMARIO

Il Glioblastoma (GBM) è un tumore cerebrale primario di IV grado (WHO 2007). Nonostante gli ultimi progressi in chirurgia, chemioterapia e radioterapia, questo tumore al cervello è ancora incurabile e recidivante (Stupp *et al,* 2009). È stato ipotizzato che la sua resistenza alle terapie e le recidive potrebbero essere dovute all'esistenza di una sottopopolazione di cellule tumorali, denominata cellule staminali di glioma (Glioma Stem Cells - GSC) (Galli *et al,* 2004; Bao *et al,* 2006). L'obiettivo della terapia del GBM è quindi il targeting selettivo delle GSCs e, tra le possibilità, l'uso di vettori virali è stata ampiamente studiato (Kroeger *et al,* 2010). Gli Herpesvirus sono stati ampiamente studiati e modificati per il trattamento del GBM (Grandi *et al,* 2009). In questo studio è stata valutata la capacità di un vettore ingegnerizzato dall' Herpesvirus Bovino di tipo 4 (BoHV-4), chiamato BoHV-4TKdsRED, di uccidere selettivamente le GSC. Il vettore esprime la Timidina Chinasi di HSV-1 e quindi può essere utilizzato con il Ganciclovir (GCV) in un protocollo di terapia genica suicida. Il suo potenziale ruolo come vettore per il trattamento del GBM è già stato dimostrato (Redaelli *et al,* 2012).

Sono stati utilizzati tre diverse colture primarie di GSC umane (GBM2, GBM4 e GBM5) e le corrispondenti cellule coltivate in siero (FBS2, FBS4 e FBS5). Sono state infettate con BoHV-4TKdsRED ed è stato testato anche un trattamento combinato del vettore con GCV sulle GSCs. È stato anche analizzato qualora vie di segnale di apoptosi o autofagia vengano attivate nelle GSCs infettate. Per verificare l'effetto di BoHV-4TKdsRED sono stati compiuti saggi di counting/killing, saggi di placca, Western Blot e analisi FACS.

BoHV-4TKdsRED è in grado di infettare le GSCs e le cellule FBS. Può replicarsi in quasi tutte le colture esaminate e le può uccidere *in vitro*. Il trattamento combinato del vettore col GCV porta ad un aumento della mortalità nelle GSCs. Segni iniziali di attivazione dell'autofagia sono stati rilevati con il trattamento combinato nelle GSCs.

Lo studio dell'apoptosi attraverso analisi FACS ha dato risultati non affidabili.

Un altro vettore derivato da BoHV-4, BoHV-4EGFPΔTK, ha dimostrato la sua capacità di infettare le cellule self-renewing, ma senza esercitare un effetto citopatico.

Secondo i nostri risultati, BoHV-4TKdsRED potrebbe essere un promettente vettore per le GSCs, ma ulteriori analisi devono essere effettuate per rafforzare questi dati.

2. INTRODUCTION

2.1 Glioblastoma

Astrocytic tumors have been classified by the World Health Organization (WHO) in four different grades, with an increasing degree of malignancy. On the basis of cytological atypia, anaplasia, mitotic activity, microvascular proliferation and necrosis we can distinguish low grade gliomas (Grade I Pilocytic astrocytoma; Grade II Diffuse Astrocytoma) and high grade gliomas (Grade III Anaplastic astrocytoma; Grade IV Glioblastoma) (Louis *et al,* 2007). So, among the astrocytic tumors, Glioblastoma (GBM) is the most frequent and most malignant primary brain tumor in adults, characterized by uncontrolled cellular proliferation, diffuse infiltration, necrosis, angiogenesis, resistance to apoptosis and genomic instability (Furnari *et al,* 2007). It has an incidence of 3-4 new cases every 100.000 people per year (WHO, IARC, 2007).

In most cases glioblastomas develop *de novo* as primary glioblastomas characterized by loss of heterozygosity (LOH) of chromosome 10q, EGFR amplification, $p16^{INK4a}$ deletion and PTEN mutations, but they can also progress from low grade astrocytomas or anaplastic astrocytomas in secondary glioblastomas with high frequency of TP53 mutations (Ohgaki *et al,* 2007).

According to genomic analyses that identified specific abnormalities in some peculiar genes, glioblastomas could be classified in 4 different subtypes with different clinical outcomes:

- Classical: characterized by chromosome 7 amplification paired with chromosome 10 loss, high level of EGFR amplification, lack of p53 mutations;
- Mesenchymal: characterized by lower NF1 expression levels, high expression of genes in the tumor necrosis factor super family and NF-κB pathway, high expression of mesenchymal markers such as CHI3L and MET;
- Proneural: defined by high levels of PDGFRA expression, point mutations in IDH1, TP53 mutations, high expression of OLIG2;
- Neural: expressing neuron markers such as NEFL, GABRA1 and SYT1 (Verhaak *et al,* 2010).

The complexity of this tumor stays beyond a large amount of genetic mutations. Among these, some were identified as prognostic and/or predictive biomarkers. The first of these is the chromosome 1p/19q codeletion, often associated with IDH1/IDH2 mutation and

exclusive from TP53 mutation and EGFR amplification. Patients with 1p/19q deletion have a better prognosis for survival compared with those of equivalent grade that lack the deletion (Ahmed *et al,* 2014). IDH1/IDH2 mutations lead to histone and DNA hypermethylation and so in a block of cell differentiation (Lu *et al,* 2012). In approximately 40% of glioblastomas the MGMT (Methyl-Guanine Methyl Transferase) promoter is methylated. This induces low expression of MGMT protein, a DNA-repair protein, and this makes cells more sensitive to alkylating agents (Hegi *et al,* 2005).

The available therapies consist in surgical resection to the extent that is safely achievable, followed by radiotherapy and adjuvant chemotherapy with Temozolomide, an alkylating agent (Stupp *et al,* 2005). Despite these combined treatments, the median survival still remains around 14.6 months after diagnosis and the average survival rate at five years is around 10% (Stupp *et al,* 2009). Thus GBM is still an incurable disease and the tumor always recurs.

2.2 Glioma Stem-Like Cells

The propagation and the resistance of GBM to therapies are thought to be driven by a subpopulation of cells, called Glioma Stem-like Cells (GSCs). Like physiological neural stem cells, GSCs display properties of self-renewal and multi-potential differentiation (Ignatova *et al,* 2002). Cancer Stem cells have been first identified in acute myeloid leukemia and breast cancer as those cells able to initiate and maintain these neoplasia (Lapidot *et al,* 1994; Bonnet *et al,* 1997; Al-Hajj *et al,* 2003). Whether cells like these were present also in brain tumors remained a mystery until Singh and colleagues in 2003 identified cells able to proliferate, self-renew and differentiate from primary human brain tumors. These cells were isolated as described by Reynolds and Weiss in 1992 to identify stem cells in adult mammalian striatum (Reynolds *et al,* 1992). The isolated cells were able to form neurosphere-like colonies, self-renew, differentiate in a phenotype identical to the tumor *in situ* and expressed the surface marker CD133, a marker for hematopoietic and neural stem cells (Singh *et al,* 2003). A simultaneous study directed by Galli and colleagues showed the presence of multipotent precursors selected from adult human brain tumors. They also demonstrated the tumorigenic potential of these cells through transplantation into immunosuppressed mice. Histologic analysis of the tumors obtained this way showed a pattern very similar to the human one from which the cells were isolated (Galli *et al,* 2004).

Moreover it has been reported that the fraction of tumor cells expressing CD133 increases after radiation in gliomas and that GSCs show an increased chemoresistance due to the overexpression of drug efflux pumps, such as ABCG2 and ABCA3 transporter proteins (Bao *et al,* 2006; Stopschinski *et al,* 2012). GSCs also possess efficient DNA-damage repair systems: for example, MGMT (Methyl-Guanine Methyl Transferase) a DNA repair gene (Hegi *et al,* 2005; Liu *et al,* 2006). These data lead to the hypothesis that Glioma Stem-like Cells might be the cause of GBM recurrence.

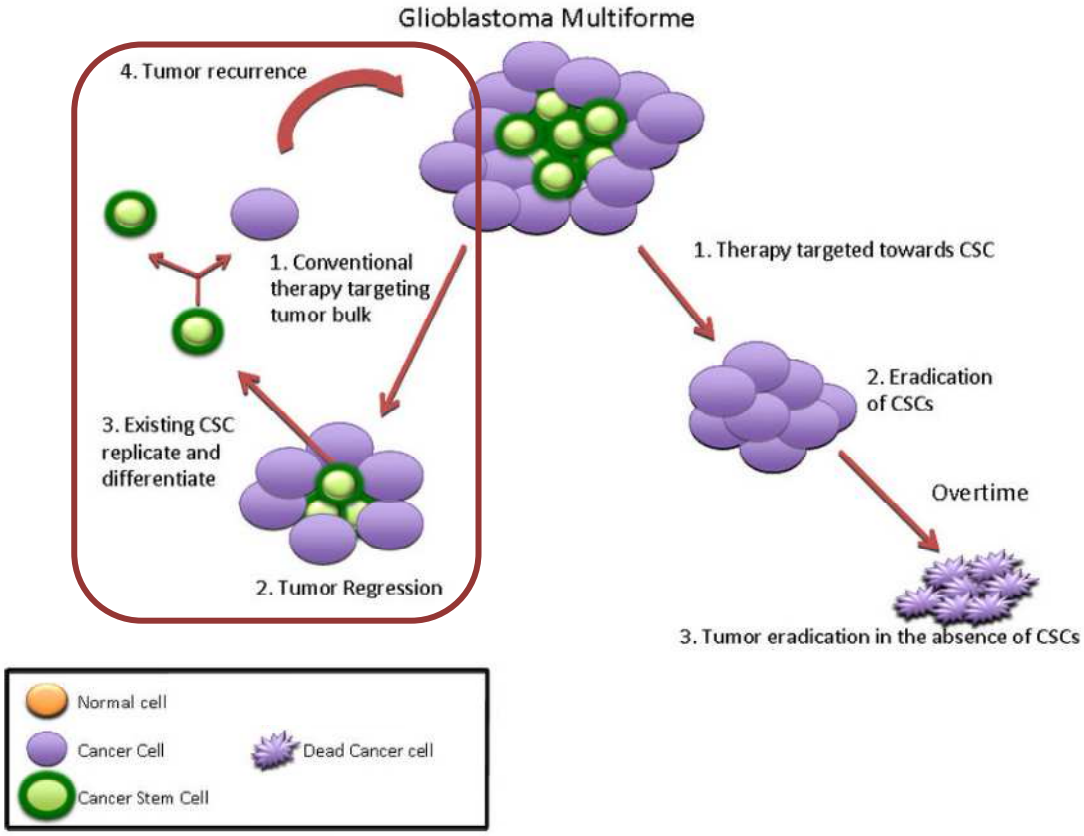

Figure 1. The Glioma Stem-like Cell Hypothesis: conventional therapy cannot eradicate the Glioma Stem Cells so the tumor recurs. The goal of novel therapies is the specific targeting of Glioma Stem Cells (modified from (Dey *et al,* 2010).

GSCs could be identified by functional criteria such as the ability to self-renew, to be multipotent and to be tumorigenic in animal models (Singh *et al,* 2003; Singh *et al,* 2004). GSCs could also be defined with molecular characteristics that are, to date, not completely understood (Bayin *et al,* 2014). There is not a specific molecular marker for GSCs but only a "stemness signature" (Shats *et al,* 2011).

The most famous stem marker, and the most debated one, is CD133, a transmembrane protein found in embryonic stem cells, adult neural stem cells and ependymal cells. Isolated CD133-positive cells resulted more tumorigenic whether injected into immunodeficient animals than CD133-negative cells (Singh *et al,* 2004), but Beier and colleagues demonstrated stem characteristics of CD133-negative cells (Beier *et al,* 2007). Another studied marker is Nestin, an intermediate filament protein in common with neural stem cells which expression is increased in GSCs and may be related to an undifferentiated state (Chen *et al,* 2012). Sox2 is a transcription factor highly expressed in GSCs and important for their maintenance (Alonso *et al,* 2011). The expression of Musashi-1, a neural RNA-binding protein, resulted higher in high grade gliomas compared to low grade ones (Toda *et al,* 2001). Other markers were investigated, but the high heterogeneity of GSCs doesn't allow to identify a universal marker.

GSCs are also influenced by the environment. Indeed, they may reside in specific niches: the perivascular niche of the tumor, where they interact with endothelial cells secreting factors useful to maintain their stemness (Calabrese *et al,* 2007). Moreover, GSCs in this niche release Vascular Endothelial Growth Factor (VEGF) contributing to neoangiogenesis (Bao *et al,* 2006). GSCs were also identified in hypoxic regions of the tumor, and *in vitro* studies demonstrated a higher clonogenicity and increased expression of stem markers in GSCs cultured under hypoxic conditions (Li *et al,* 2009; Bar *et al,* 2010).

2.3 Gene Therapy And Virotherapy Against GBM

So different strategies can be used to target GSCs and, among these, viruses can be used in two different methods. The first one is the virotherapy, where viral vector replication induces lysis of the neoplastic cells. This can be achieved through a selective infection of cancer cells by the vectors used, sparing the brain parenchyma. The other one is the gene therapy, where viral vectors are carriers of transgenes able to correct mutation, to stimulate immunity or to activate prodrugs in cancer cells (Dey *et al,* 2010; Kroeger *et al,* 2010).

GBM is a tumor with a peculiar localization and a low tendency to metastasize. These characteristics are favorable for the virotherapy because viral vectors can be stereotactically injected at the tumor site, avoiding systemic toxic events (Pulkkanen *et al,* 2005).

Engineered adenoviruses (Chiocca *et al,* 2004; Nandi *et al,* 2009), polioviruses (Goetz *et al,* 2010), reoviruses (Kicielinski *et al,* 2014), retroviruses (Tai *et al,* 2008), measles virus (Allen *et*

al, 2013) and herpesviruses (Cassady *et al,* 2010; Wollmann *et al,* 2012; Ning *et al,* 2014) have been largely studied as oncolytic vectors.

Among these, Human Herpesvirus type 1 (HSV-1) has been largely used in the latest 25 years, exploiting its large genome (152kb) which allows an insertion up to 30-50 kb transgenes (Bleiziffer *et al,* 2007). Due to its neurotropism and neurotoxicity it has been necessary to delete its neurovirulence genes to permit replication only in dividing tumor cells, avoiding infection of the normal brain tissue and lethal encephalitis (Kennedy *et al,* 1993; Mineta *et al,* 1995). Since 1991, when the first oncolytic HSV-1-thymidine kinase-deficient mutant has been used to kill U87 glioma cells, different generations of oncolytic HSV have been engineered (Martuza *et al,* 1991; Cassady *et al,* 2010; Wollmann *et al,* 2012). Among these, G207 and G47Δ have also been used to target glioma stem-like cells. G47Δ showed a more efficient killing of GSCs than G207, a better reduction of GSCs self renewal and *in vivo* a considerable inhibition of tumor growth in tumor-bearing immunodeficient mice (Wakimoto *et al,* 2009; Sgubin *et al,* 2012).

HSV-1 has been also used in protocols of the so called Suicide Gene Therapy (SGT). This type of therapy consists in the conversion of prodrugs in toxic metabolites only in those cells that express the gene capable to do this conversion (Iwami *et al,* 2010). Together with cytosine deaminase that converts the prodrug 5-fluorocytosine in the toxic metabolite 5-fluorouracil, HSV-1 Thymidine kinase gene is the most common gene used in protocols of SGT. HSV-1 TK phosphorylates the prodrug Ganciclovir in its triphosphate active compound which inhibits DNA synthesis leading to cell death, with 1000-fold greater efficiency than mammalian thymidine kinases (Altaner 2008). This method has been tested with different vectors in many pilot studies and, to date, in 16 clinical trials with controversial results (Barzon *et al,* 2006; Kaufmann *et al,* 2014).

In recent years the idea to combine gene therapy or virotherapy with immunotherapy, to actively stimulate an anti-tumor immune response, has become increasingly marked (Ning *et al,* 2014). Already in 1999 a pilot study in 4 GBM patients with a retroviral vector expressing HSV-1 TK and the cytokine gene IL-2 demonstrated an anti-tumor activity and a subsequent clinical trial in an enlarged population of patients (n=12) showed positive responses, in terms of safety and regression of tumor masses, in 50% of the cases (Palu *et al,* 1999; Colombo *et al,* 2005). More recently, an oncolytic HSV-1 (G47Δ) armed with IL-12 has been tested in a

new mouse GSC-derived tumor model showing reduction in tumor growth and prolonged survival in treated animals (Cheema *et al,* 2013).

In the present study a viral vector obtained from the Bovine Herpesvirus type 4 has been used.

2.4 Bovine Herpesvirus Type 4 As A Viral Vector In GBM Therapy

Bovine Herpesvirus type 4 (BoHV-4) belongs to the *Gammaherpesvirinae* subfamily and to the *Rhadinovirus* genus and has a linear double stranded DNA of approximately 145 kb containing a unique central sequence of 110 kb with low G-C content, flanked by polyrepetitive DNA (prDNA) of high G-C content and variable size. According to their restriction patterns, BoHV-4 strains can be divided in two groups: the Movar 33/63-like and the DN 599-like (Bublot *et al,* 1990). Its genome encodes at least 79 Open Reading Frames (ORFs), some of which are homologues to ORFs presents in others Herpesviruses (Palmeira *et al,* 2011). Like other Herpesviruses, BoHV-4 virions are composed of a nucleocapsid surrounded by the tegument and enclosed by a lipid envelope forming infectious virus particles with a diameter around 200 nm. BoHV-4 envelope has ten glycoproteins, among which glycoproteins gB, gH, gL, gM and gN are conserved in all Herpesviruses (Dubuisson *et al,* 1989). Moreover herpesvirus virions are composed also of a variety of host proteins (Lete *et al,* 2012). Glycoproteins are involved in virus attachment, penetration, budding and spreading among infected cells.

Herpesviruses use a core fusion machinery composed of gB and the gH/gL heterodimer to enter the cells, but the mechanism is not still completely understood for all Herpesviruses. Recently it has been demonstrated that BoHV-4 glycoprotein L is not essential for membrane fusion, but its lack causes impaired cell penetration leading to virus growth deficit (Lete *et al,* 2012). Moreover BoHV-4 glycoprotein B, one of the most conserved among the herpesvirus family, has been shown to be indispensable for virus replication (Franceschi *et al,* 2013).

BoHV-4 has not a clear pathogenic role; it has been isolated from samples and cells of healthy cattle or cattle with various clinical symptoms such as skin lesions, respiratory diseases or metritis (Dubuisson *et al,* 1989). It establishes persistent infections of the monocyte/macrophage lineage in its natural host and in an experimental one, the rabbit (Osorio *et al,* 1982; Osorio *et al,* 1983). It has been demonstrated that BoHV-4 can replicate and causes cytopathic effect (CPE) in a variety of immortalized cell lines and primary cell

cultures (Peterson *et al,* 1988; Donofrio *et al,* 2002). In recent years a recombinant BoHV-4 expressing Enhanced Green Fluorescent Protein (EGFP) (BoHV-4EGFPΔTK) has been proposed as a gene delivery vector. Donofrio and colleagues demonstrated its ability to infect cell lines from different species with or without CPE, viral replication and EGFP expression. Further, they proved that it is able to cause a persistent infection in neuronal cells (Donofrio *et al,* 2002). In vivo experiments showed that the intranasal inoculation in mice of BoHV-4EGFPΔTK doesn't cause neuroinvasion or systemic infection and its injection in the mouse lateral ventricle carries out EGFP expression in the ependymal cells and in GFAP-positive cells of the rostral migratory stream (RMS), without showing any viral replication in the mouse brain (Donofrio *et al,* 2006).

More recently another vector, BoHV-4-A-TK^{HSV-1}-IRES-dsRed (hereafter referred as BoHV-4TKdsRED), has been obtained by Prof. Donofrio as follows: the HSV-1-TK Open Reading Frame was cut from the pHyTK plasmid vector, subcloned in pIRES2DsRED-Express2 vector and linearized with NheI/SmaI restriction enzymes to generate the phCMVie-TK^{HSV-1}-IRES-dsRed-pA vector. Then the TK^{HSV-1}-IRES-dsRed module was cut and subcloned in the pINT2EGFPTK vector (Donofrio *et al,* 2002), depriving it of the GFP ORF, and obtaining this way the pTK-hCMVie-TK^{HSV-1}-IRES-dsRed-pA-pTK construct. The purified gel fragment TK-hCMVie-TK^{HSV-1}-IRES-dsRed-pA-TK was then electroporated into SW102 bacteria with KanaGalK targeted into the BoHV-4-A thymidine kinase locus. After selective growth, the BAC-BoHV-4-A was purified and analyzed for TK-hCMVie-TK^{HSV-1}-IRES-dsRed-pA-TK fragment integration. After genome characterization, the pBAC-BoHV-4-A- hCMVie-TK^{HSV-1}-IRES-dsRed-pA plasmid DNA has been electroporated into BEK or BEK*cre* cells and the recombinant virus expanded in MDBK cells. The engineered vector obtained this way expresses *HSV-1 TK* and the Red Fluorescent Protein as a reporter gene. The expression of *HSV-1 TK* allows the infected cells to interact with the prodrug Ganciclovir (GCV) in a protocol of Suicide Gene Therapy.

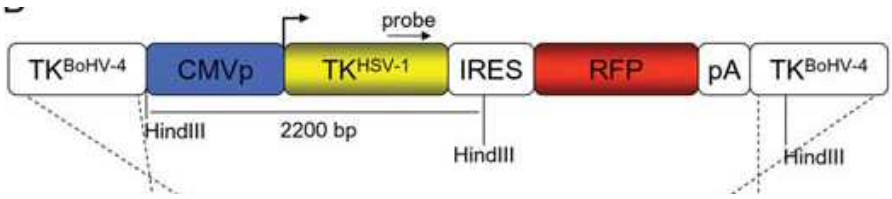

Figure 2. Transgene scheme of BoHV-4TKdsRED (modified from (Redaelli *et al,* 2012)

BoHV-4TKdsRED resulted able to infect immortalized glioma cell lines of rats (F98), mice (GL261) and humans (GLI36) and a primary culture of human glioblastoma. Redaelli and colleagues demonstrated that the combined treatment with GCV enhances the cytopathic effect of the vector and increases apoptotic cell death. *In vivo* experiments performed on mice and rats stereotactically injected with syngeneic glioma cells and subsequently treated with BoHV-4TKdsRED and/or GCV demonstrated an increased survival in animals treated with BoHV-4TKdsRED in combination with GCV (Redaelli *et al,* 2012).

2.5 Programmed Cell Death In Cancer

The aim of an anti-cancer therapy is neoplastic cells death. However, these cells often have alterations of signaling pathways of programmed cell death, such as apoptosis and autophagy.

Apoptosis is characterized by biochemical and morphological changes, such as blebbing, anoikis and nuclear condensation and fragmentation. Altered expression of some key factors of apoptosis pathways may contribute to cancer formation (Ouyang *et al,* 2012). Anti-apoptotic proteins are upregulated in GBM, together with downregulation of pro-apoptotic proteins (Krakstad *et al,* 2010).

Autophagy can act a pro-survival, in cell homeostasis, or a pro-death role, if cells are exposed to an excessive stress. It is characterized by the formation of autophagosomes that surround cytoplasmic macromolecules and organelles. In early stages of cancer autophagy can have a protective role, but can also have a pro-tumor role (Ouyang *et al,* 2012). The autophagic capacity is downregulated in GBM, indeed the reduced expression of autophagic proteins positively correlates with the tumor progression (Huang *et al,* 2010).

It has been described that HSV-1 infection can induce autophagy or apoptosis in U251 glioma cell line; moreover autophagy can counteract apoptosis, playing this way a pro-survival role in infected cells (Tovilovic *et al,* 2013). γ34.5 gene of HSV-1 blocks autophagy during infection through its binding to Beclin 1, so γ34.5-deleted HSV-1 vectors have a more pro-autophagic effect in infected cells than wild type virus (Cavignac *et al,* 2010; Kanai *et al,* 2012).

Sciortino and colleagues showed that BoHV-4 causes apoptotic cell death in a permissive cell line (BS/BEK cells) (Sciortino *et al,* 2000) and recently autophagy induction has been demonstrated in MDBK cells after BoHV-4 infection (Montagnaro *et al,* 2013).

3. AIM OF THE STUDY

Starting from the published results obtained in glioma cell lines of rats, mice and humans infected with BoHV-4TKdsRED (Redaelli *et al,* 2012), the main aim of the present study is to verify whether the vector is able to infect GSCs, to replicate in them and to kill them. Further, the present study aims to study BoHV-4TKdsRED effect in combination with the prodrug Ganciclovir (GCV), with a particular attention on the possible mechanisms involved.

4. MATERIALS AND METHODS

4.1 Cell Culture

Human GSCs primary cultures (GBM2, GBM4 and GBM5) derived from human GBM surgical specimens have been previously established, following published protocols (Wakimoto *et al,* 2009; Sgubin *et al,* 2012). Surgical specimens were obtained from a 58-years-old man patient (GBM2), a 58-years-old woman patient (GBM4) and a 63-years-old woman patient (GBM5). Cells have been cultured as spheres in EF20 medium: Neurobasal medium (Life Technologies Italia, 21103049; http://www.lifetechnologies.com) supplemented with 3 mmol/l L-Glutamine (Sigma-Aldrich Chemie GmbH, G6392-1VL; http://www.sigmaaldrich.com), 1X B27 supplement (Life Technologies Italia, 17504044), 0.5X N2 supplement (Life Technologies Italia, 17502048), 2 µg/ml heparin (Sigma-Aldrich Chemie GmbH, 84020), 0.5X Penicillin, Streptomycin/Amphotericin B complex (Euroclone S.p.A, ECM0010D.; http://www.euroclone.it), 20 ng/ml recombinant human EGF (R&D System Inc., 236-EG-200; http://rndsystems.com) and 20 ng/ml recombinant human FGF2 (Peprotech, 100-18B-50UG; http://www.peprotech.com). Cell passaging was performed by chemical dissociation of spheres using the NeuroCult^{TM} Chemical Dissociation kit (StemCell Technologies, 05707; http://www.stemcell.com).

Part of the GBM2, GBM4 and GBM5 specimens were digested by trypsin-EDTA 0.05% (Life Technologies Italia, 25300-054) and then cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies Italia, 41965039) supplemented with 10% fetal bovine serum (FBS) (Life Technologies Italia, 10270-106), 1X Penicillin/Streptomycin Solution (Euroclone S.p.A., ECB3001D), 10 µg/ml Tetracycline Hydrochloride (Sigma-Aldrich Chemie GmbH, T7660-5G) and 35 ng/ml Mycoplasma Removal Agent (Euroclone S.p.A., ECMC210A) to generate adherent cultures (FBS2, FBS4 and FBS5). For these cultures passaging was performed by trypsinization.

The cells were cultured under normoxic conditions (5% CO_2, 21% O_2) at 37°C in humidified incubators. GSCs cultures were maintained until 60 passages before freezing; serum-cultured cells were maintained until 30 passages before freezing.

4.2 Virus

BoHV-4EGFPΔTK and BoHV-4TKdsRED were kindly provided by Prof. G. Donofrio (University of Parma).

BoHV-4EGFPΔTK derives from Bovine Herpes Virus type 4 and was obtained by homologous recombination of the viral genome with the construct pINT2EGFPTK (Donofrio *et al,* 2002).

BoHV-4TKdsRED was obtained by homologous recombination of the viral genome with the construct pTK-hCMVie- TK^{HSV-1}-IRES-dsRed-pA (Redaelli *et al,* 2012). The transgene is inserted in the deleted Thymidine Kinase (TK) locus, thus, the engineered vector expresses *HSV-1 TK* and the Red Fluorescent Protein as a reporter gene. The expression of *HSV-1 TK* allows the infected cells to interact with the prodrug Ganciclovir in a protocol of Suicide Gene Therapy.

Human Herpes Simplex Virus type 1 (HSV-1) strain F was kindly provided by Dr. A. Calistri (University of Padua).

4.3 Initial Virus Titration

To assess the initial titer of BoHV-4TKdsRED a classical Plaque Assay was performed on Vero Cells, plated at a density of $2*10^5$ cells/well in 6-well plates. Serial dilutions (from 10^{-6} to 10^{-11}) of the viral stock were performed in PBS/1%IFBS (Inactivated FBS). 700 µl of each diluted sample were used to infect the cells. After 120 minutes of incubation at 37°C, the viral inoculum was removed and replaced with DMEM, DMEM/10%FBS and methylcellulose (respectively 55%, 20% and 25%); cells were then incubated at 37°C until plaques achieved an adequate size (4 days). The medium was then removed and cells were fixed with methanol and stained with Giemsa. Plaques were counted and the viral titer was calculated.

The initial titer of HSV-1 has been performed as described for BoHV-4TKdsRED.

4.4 Cell Growth Curves

GBM2, GBM4 and GBM5 spheres were dissociated and $2*10^4$ cells/well were plated in 24-well plates; FBS2, FBS4 and FBS5 confluent cells were trypsinized and plated at the same density in 24-well plates. On days 3, 6 and 9 cells were collected and counted on a Burker chamber with Trypan Blue. Trypan Blue-excluding cells were counted as viable cells. Each condition was performed in triplicate.

4.5 Self-Renewal Assay

GBM2, GBM4 and GBM5 spheres were dissociated with NeuroCult™ Chemical Dissociation kit and cells passed through a 40 µm cell strainer (Stem Cell Technology, 27305) to obtain a single cell suspension. Cells were then seeded in uncoated 96-wellplates at two dilutions (10 and 1 cells/well) in 100 µl of EF20 medium. Cells were fed weekly with 100 µl/well of fresh medium and spheres counted when a size of 80-100 µm was achieved. Experiments were performed three times.

4.6 Virus Yield Assay

GBM2, GBM4 and GBM5 spheres were dissociated, $2*10^4$ cells/well were plated in 24-well plates and infected with BoHV-4TKdsRED at a Multiplicity Of Infection (MOI) 1. At 12, 24, 48 and 72 hours after infection, cells and supernatants were harvested and processed with three freeze/thaw cycles and sonication. The viral titer was determined by Plaque Assay on Vero cells as already described. The same experiment was performed with the FBS cultures. For GBM5 and FBS5 cells the experiment was extended to 144 and 216 hours after infection. Each condition was performed in triplicate.

4.7 Cell Killing Assay

GBM2, GBM4 and GBM5 spheres were dissociated and plated at a density of $2*10^4$ cells/well in 24-well plates and infected with mock or with BoHV-4TKdsRED at MOI 0.5; 3, 6 and 9 days after infection cells were trypsinized to dissociate the spheres. Viable Trypan Blue-excluding cells were counted on a Burker chamber. The Fraction Affected (Fa) has been calculated following the formula: Fa= (1- # alive cells with virus)/average alive mock cells. The same experiment was performed with the FBS cultures.

The same experiment was performed with GBM2, GBM4 and GBM5 cells with HSV-1.

Each condition was performed in triplicate.

4.8 Suicide Gene Therapy Protocol For Cell Killing Assay

GBM2, GBM4 and GBM5 spheres were dissociated and plated at a density of $2*10^4$ cells/well in 24-well plates. Cells were then infected with mock or BoHV-4TKdsRED at MOI 0.5 and 24

hours after infection treated with different concentrations of Ganciclovir GCV (Sigma-Aldrich Chemie GmbH, G2536) (0, 10, 50 and 100 µg/ml). After 3, 6 and 9 days cells were trypsinized to dissociate the spheres and cells were counted after Trypan Blue on a Burker chamber. Each condition was performed in triplicate.

4.9 Western Blots

GBM2, GBM4, GBM5, FBS2, FBS4 and FBS5 untreated cells were seeded at a density of $5*10^5$ in Petri dish and after some days collected and centrifuged at 405 rcf for 5 minutes. The pellet was resuspended with PBS and centrifuged again. Cells were then resuspended in RIPA buffer (Sigma-Aldrich Chemie GmbH, R0278) with protease inhibitors (Roche, 04693116001; http://www.roche.com), incubated in ice for 30 minutes and centrifuged at 11800 rcf for 10 minutes. The supernatant lysates were collected and stored at -80°C. Protein concentrations were determined by BCA Protein Assay (Micro BCA Protein Assay kit, Thermo Scientific Pierce, 23235; http://www.piercenet.com). Proteins were separated on a 7.5%, 10% or 12% SDS-PolyAcrylamide gels, transferred to nitrocellulose membrane (GE HealthCare, RPN303D; http://www.gelifesciences.com). Blocking was performed with 5% nonfat dry milk (Bio-Rad, 170-6404; http://www.bio-rad.com) or Bovine Serum Albumin (BSA) (Sigma-Aldrich Chemie GmbH, A9647) in Tris-Buffered Saline and Tween 20 (TBST) 0.1% for 45 minutes at room temperature. Membranes were then incubated overnight with primary antibodies at 4°C. The next day membranes were washed tree times with TBST 0.1% (10 minutes each time). Membranes were then incubated with the corresponding secondary peroxidase-conjugated antibody for 1 hour at room temperature and washed four times with TBST 0.1% (10 minutes each time). Proteins were then visualized with ECL Western blotting detection kit (GE HealthCare, RPN2106) on Kodak films (Sigma-Aldrich Chemie GmbH, Z370371-50EA).

The primary antibodies used were as follows: 1:5000 goat anti-Nestin (SCBT, sc21247); 1:1000 rabbit anti-Sox2 (Cell Signaling, 3579); 1:1000 mouse anti-α-tubulin (Sigma-Aldrich Chemie GmbH, T5168); 1:1000 mouse anti-GFAP (Sigma-Aldrich Chemie GmbH, G3893); 1:500 rabbit anti-VEGF (SCBT, sc152); 1:1000 rabbit anti-βIII-tubulin (Covance, MRB-435P)

The secondary HRP-conjugated antibodies were diluted in 5% nonfat dry milk in TBST 0.1% as follows: 1:2000 anti-rabbit IgG (GE HealthCare, RPN4301), anti-mouse IgG (GE HealthCare, RPN4201) and anti-goat IgG (Chemicon, API06P).

GBM2 cells were seeded at a density of $5*10^5$ cells/well in a 6-wellplate and the day after infected with mock or BoHV-4TKdsRED at MOI 1. After 24 hours cells were treated or not with GCV at a concentration of 50 µg/ml and the day after cells were collected and proteins extracted as described above.

GBM2 and GBM5 cells were seeded as already described and the day after treated with BoHV-4TKdsRED at MOI 1, Rapamycin (SCBT, sc3504) 1 µM or 2 µl of DMSO. After 24 hours cells were treated or not with GCV 50 µg/ml. Cells were collected at different time points: 24, 48 and 72 hours post BoHV-4TKdsRED infection; 24 and 48 hours post GCV treatment; 24, 48 and 72 hours post Rapamycin treatment.

Western Blots were performed as depicted above and additional primary antibodies were used: 1:1000 rabbit anti-p62 (Sigma-Aldrich Chemie GmbH, P0067); 1:1000 rabbit anti-LC3 (Sigma-Aldrich Chemie GmbH, L7543).

4.10 FACS Analysis

GBM2, GBM5 and FBS5 cells were seeded at a density of $2.5*10^5$ cells/well in a 6-wellplate and the day after treated with mock or BoHV-4TKdsRED at MOI 1, with Paclitaxel (Sigma-Aldrich Chemie GmbH, T7191-1MG) 100 nM or 2 µl of DMSO. 24 hours later cells were treated or not with GCV 50 µg/ml. GBM2 and GBM5 cells were collected 72 hours after BoHV-4TKdsRED infection, Paclitaxel or DMSO treatment, pelleted and dissociated with 100 µl of Versene. FBS5 cells were washed once with PBS and trypsinized. Versene or trypsin were inactivated with DMEM 10% FBS. Cells were centrifuged, pellets washed twice with 1 ml of PBS and resuspended in Annexin V Binding Buffer (BD Pharmingen™, 556454; http://www.bdbiosciences.com). Cells were stained with FITC-Annexin V (BD Pharmingen™, 556420) and Propidium Iodide (Sigma-Aldrich Chemie GmbH, P4170) following the manufacturer protocol. Samples were then analyzed using a FACScalibur™ (BD Biosciences; http://www.bdbiosciences.com).

4.11 BoHV-4EGFPΔTK Infection Of GSCs

$2.5*10^4$ GBM5 cells were plated and the day after infected with BoHV-4EGFPΔTK at MOI 0.2. The infection was monitored through EGFP expression under epifluorescence microscope (Leica DMIL LED). After 7 days of infection cells were dissociated with NeuroCult™ Chemical Dissociation kit and plated 1 cell/well in uncoated 96-wellplate. Cells were fed weekly with

100 µl/well of fresh medium until spheres formation. Spheres were collected, mechanically dissociated and separately transferred in T25 flasks. Cells were then monitored until 60 days after infection.

4.12 Statistical Analysis

Differences in cell growth, killing assay and suicide gene therapy protocol were compared using a two-tailed unpaired t-test. p values <0.5 and p<0.01 were considered significant using GraphPad (GraphPad Software; http://www.graphpad.com) and Excel (Microsoft; http://www.microsoft.com).

5. RESULTS

5.1. Cell Characterization

5.1.1 Cell Growth

The use of primary cultures implicates the necessity to study how the cells grow. To determine the GSCs growth, GBM2, GBM4 and GBM5 cells were dissociated, $2*10^4$cells/well were plated and counted at day 3, 6 and 9 after plating. As expected each GSCs culture showed different growth rates.

In 9 days GBM2 cells increased five-fold compared to their initial number (Figure 3A). Significant differences have been calculated between day 0-day 3 and day 3-day 6 (p=0.0156 and p=0.0126 respectively; unpaired t-test; n=9).

GBM4 cells increased four-fold their number after 9 days (Figure 3B), showing significant differences between day 3-day 6 and day 6-day 9 (p=0.0132 and p=0.0056 respectively; unpaired t-test; n=9).

The number of GBM5 cells at day 9 was almost seven-fold compared to their initial one (Figure 3C). In this case significant differences have been calculated between each time point (p=0.0062, p=0.0003 and p=0.0391 respectively; unpaired t-test; n=9).

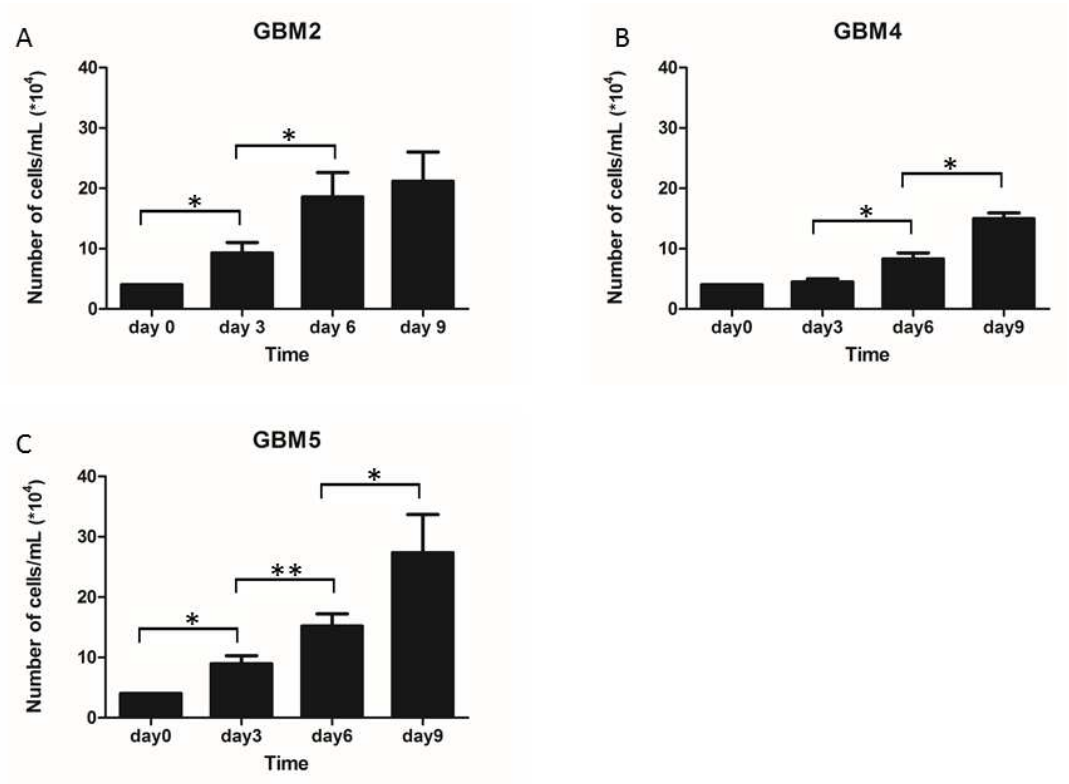

Figure 3. GBM2 (A), GBM4 (B) and GBM5 (C) growth curves. (*p<0.05; ** p<0.001; mean ± SD)

The same experiment was performed with FBS2, FBS4 and FBS5 cells. FBS2 cells reached after 9 days a cell number five-fold the initial cell number (Figure 4A), with significant differences between day 0- day 6 and day 0-day 9 ($p=0.0136$ and $p=0.0302$ respectively; unpaired t-test; n=6).

FBS4 cells achieved at day 9 a cell number twenty-fold higher compared to the cell number at day 0 (Figure 4B). A significant difference have been calculated between day 0-day 3 ($p=0.0215$; unpaired t-test; n=3).

FBS5 cells reached at day 9 a cell number twenty-fold their initial one (figure 4C), with significant differences calculated between each time point ($p=0.02$, $p=0.012$ and $p=0.0033$ respectively; unpaired t-test; n=6).

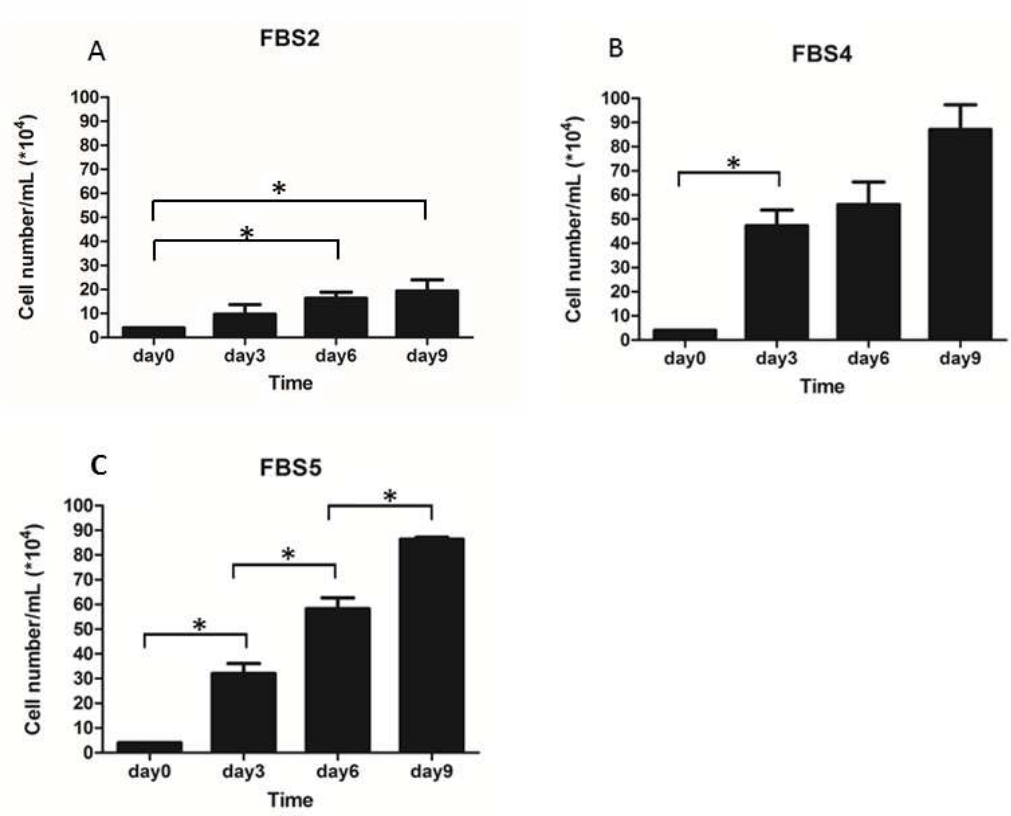

Figure 4. FBS2 (A), FBS4 (B) and FBS5 (C) growth curves. (*p<0.05; ** p<0.001; mean ± SD)

From these results, it is possible to infer that GBM2, GBM4 and GBM5 cells have a gradual time-dependent growth. Except for FBS2 cells, with a growth rate comparable to GBM2 cells, FBS4 and FBS5 cells showed a huge growth, faster than their stem counterpart, with a very rapid replication especially in the first 3 days.

These results are consistent with the published data, showing that GSCs have a low replication rate (Bao *et al,* 2006).

5.1.2 Self-Renewal Assay

One of the peculiar characteristics of GSCs is their self-renewal capability, so the ability of GBM2, GBM4 and GBM5 cells to form spheres has been tested. Single-cell suspensions were plated at clonogenic densities, 1 cell/well and 10 cell/well, in 100 µl and spheres were counted when a diameter of 80-100 µm was reached.

Almost 20% of GBM2 cells can self renew (Figure 5A); in GBM4 cells 12% can form spheres (Figure 5B); GBM5 cells showed the highest percentage of self renewing cells among our cultures, 28% of cells can form spheres (Figure 5C).

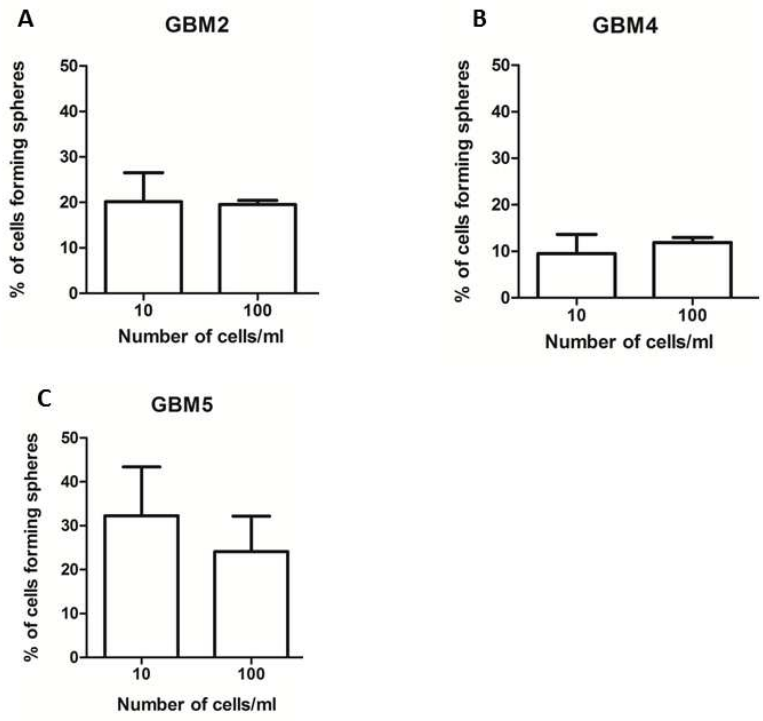

Figure 5. Percentage of cells forming spheres in GBM2 (A), GBM4 (B) and GBM5 (C). (mean ± SD)

5.1.3 Markers Expression

The expression of stem markers as Nestin and Sox2 in GSCs is well described in literature (Alonso *et al,* 2011; Chen *et al,* 2012). In order to characterize our cultures we tested the expression of Nestin, Sox2, the neuronal marker βIII-tubulin and the glial fibrillary acidic protein GFAP.

Nestin was expressed in GBM2, GBM4 and GBM5 cells and very low expressed in FBS2 and FBS4 cells; Sox2 was expressed in GBM2, GBM4 and GBM5 cells but not in FBS cultures; βIII-tubulin was expressed in GBM2, GBM4, FBS2 and FBS4 cells, but not in GBM5 and FBS5 cells;

GFAP was expressed in GBM2 cells and a very high expression has been observed in GBM4 cells. α-tubulin was used as loading control (Figure 6).

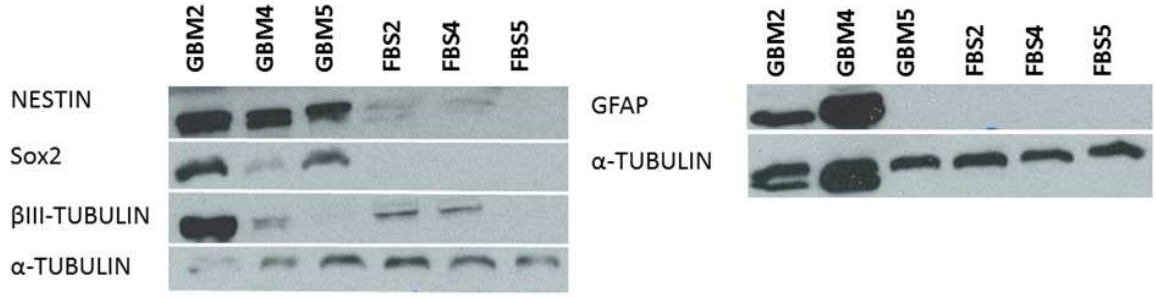

Figure 6. Western Blot performed on GBM2, GBM4, GBM5, FBS2, FBS4 and FBS5 lysates showing cell markers expression. The lowest band in α-tubulin in the right panel is due to incomplete stripping of GFAP.

5.2. BoHV-4TKdsRED Infection

2.1 Initial Virus Titration

After Plaque Assay on Vero cells with serial dilutions of BoHV-4TKdsRED, plaques were counted and the titer of the viral stock obtained was $3*10^8$pfu/ml.

The titer of the viral stock of HSV-1 obtained in the same way was $3*10^7$pfu/ml.

5.2.2 BoHV-4TKdsRED Replication In GSCs And FBS Cultures

The replication ability of BoHV-4TKdsRED has been analyzed through viral titer quantification of GBM2, GBM4 and GBM5 cells supernatants collected 12, 24, 48 and 72 hours after infection at MOI 1.

BoHV-4TKdsRED can weakly replicate in GBM2 cells (Figure 7A); the viral titer calculated 72 hours after infection was slightly higher compared to the initial input (input $4*10^4$pfu/ml; 72 hours after infection $4.5*10^5$pfu/ml). Also in GBM4 cells BoHV-4TKdsRED can slightly replicate (Figure 7B); the calculated viral titer 72 hours after infection was $2.55*10^5$pfu/ml. In GBM5 cells BoHV-4TKdsRED can't replicate within 72 hours of infection; the calculated viral titer at this time point ($5.71*10^3$pfu/ml) was lower than the initial input. Since the classical shape of a viral replication was observed, the experiment was extended till 144 and 216 hours after infection, to test whether the viral replication needs more time in these cells. The viral titers obtained at these time points was $1.41*10^5$pfu/ml and $1.17*10^5$pfu/ml

respectively at 144 and 216 hours after infection (Figure 7C). So BoHV-4TKdsRED replicates more slowly in GBM5 cells than in our others GSCs cultures.

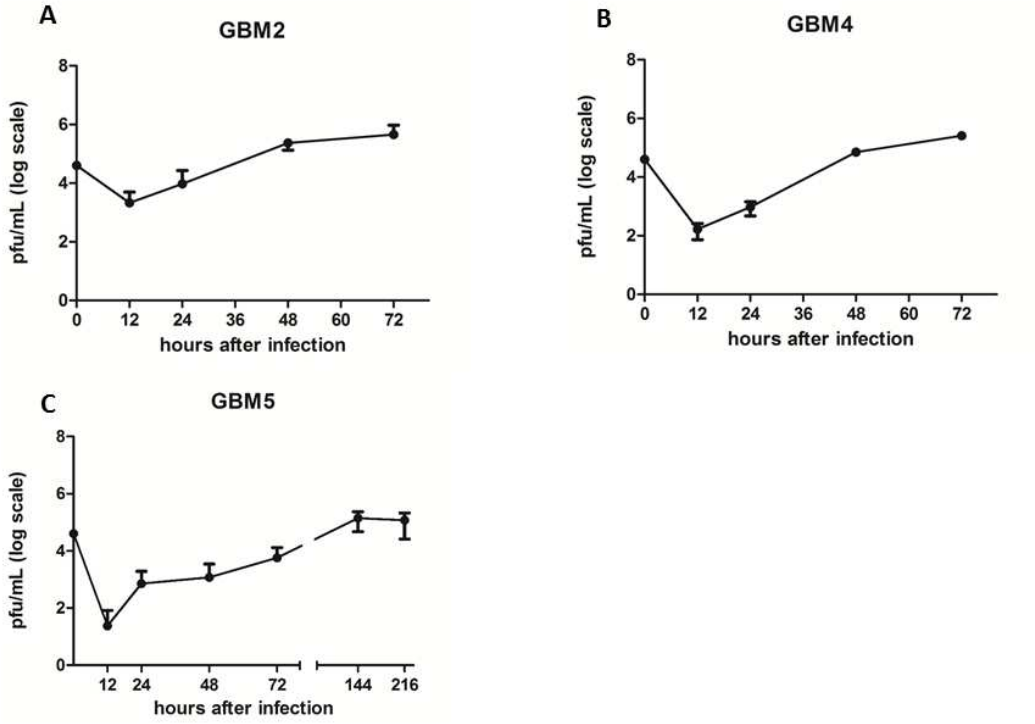

Figure 7. Replication curve of BoHV-4TKdsRED in GBM2 (A), GBM4 (B) and GBM5 (C). (mean ± SD)

The same experiment has been performed on FBS cultures. BoHV-4TKdsRED can slightly replicate in FBS2 cells (Figure 8A); after 72 hours of infection the viral titer calculated was $1.72*10^5$pfu/ml. The virus can replicate in FBS4 cells (Figure 8B); the viral titer 72 hours after infection reached $1.9*10^7$pfu/ml. As in their stem counterpart BoHV-4TKdsRED cannot replicate in FBS5 cells within 72 hours (Figure 8C). The calculated viral titer at this time point was $2.52*10^4$pfu/ml, so the experiment was extended, but even 144 and 216 hours after infection the viral titer resulted lower than the initial input (144 hours after infection $1.22*10^4$pfu/ml; 216 hours after infection $3.1*10^3$pfu/ml). Probably the virus kills the cells before completing the viral replication cycle.

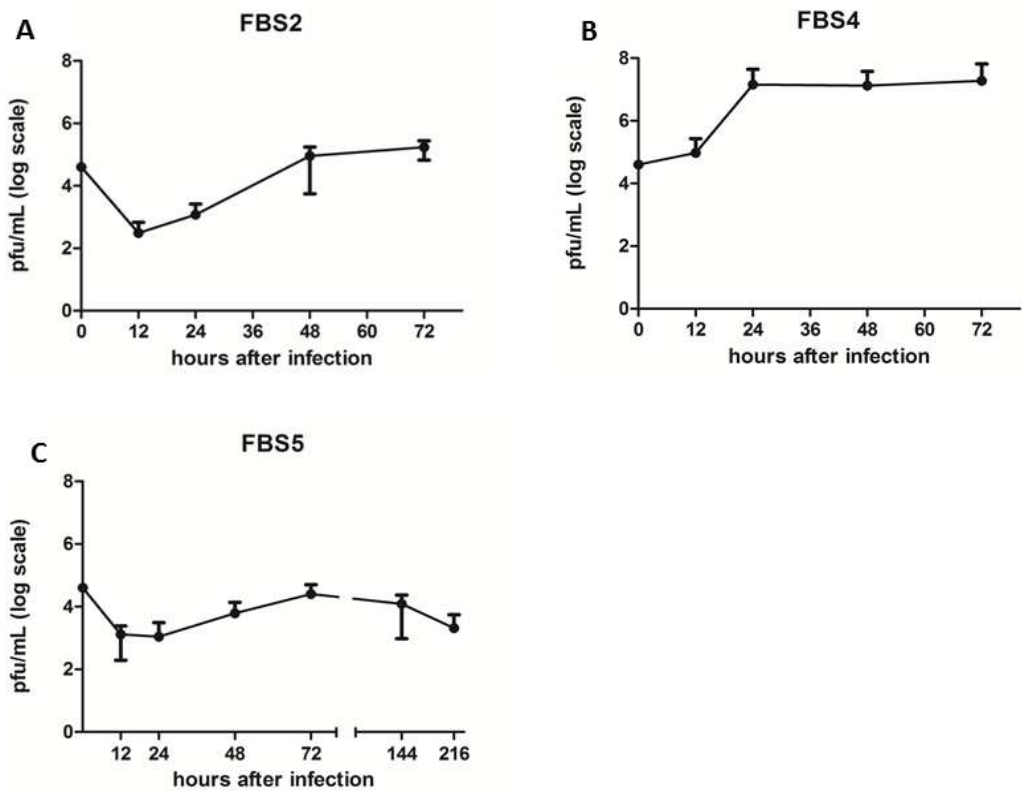

Figure 8. Replication curve of BoHV-4TKdsRED in FBS2 (A), FBS4 (B) and FBS5 (C). (mean ± SD)

5.2.3 Killing Effect Of BoHV-4TKdsRED In GSCs And FBS Cultures

One of the main aim of the project is the investigation about the killing ability of BoHV-4TKdsRED on GSCs. To check this ability, GBM2, GBM4 and GBM5 cells were plated at a density of $2*10^4$cells/well and the day after were infected with mock or BoHV-4TKdsRED (MOI 0.5). After 3, 6 and 9 days of infection cells were counted. The Fraction affected (Fa) has been calculated as a ratio between the number of cells infected in each well and the average number of mock cells in 3 wells for each time point, using the following formula: Fa= (1 - #alive cells with BoHV-4TKdsRED) / average alive mock cells.

GBM2 cells were killed by BoHV-4TKdsRED (Figure 9A): 9 days after infection 70% of cells were affected with a significant difference between day 6-day 9 (p=0.0253; unpaired t-test; n=6).

At day 9 87% of GBM4 cells were affected (Figure 9B). Significant differences have been calculated between day 3-day 6 and day 6-day 9 (p=0.0134 and 0.0013 respectively; unpaired t-test; n=3).

BoHV-4TKdsRED can kill also GBM5 cells (Figure 9C). At day 9 the percentage of cells affected was 67%, with a significant difference between day 3-day 6 (p=0.0083; unpaired t-test; n=6).

The same experiment has been performed with our FBS cultures. FBS2 cells were almost completely killed by BoHV-4TKdsRED after 9 days (98% of cells affected) (Figure 9D), with a significant difference between day 3-day 6 ($p=0.0039$; unpaired t-test; n=3).

Also 98% of FBS4 cells were affected after 9 days of infection (Figure 9E) and significant differences have been calculated between day 3-day 6 and day 6-day 9 ($p<0.0001$ and $p=0.0013$ respectively; unpaired t-test, n=3).

FBS5 cells were less sensitive to BoHV-4TKdsRED than other FBS cultures (Figure 9F). 9 days after infection 72% of cells were affected with a significant difference between day 6-day 9 ($p=0.0032$; unpaired t-test; n=3).

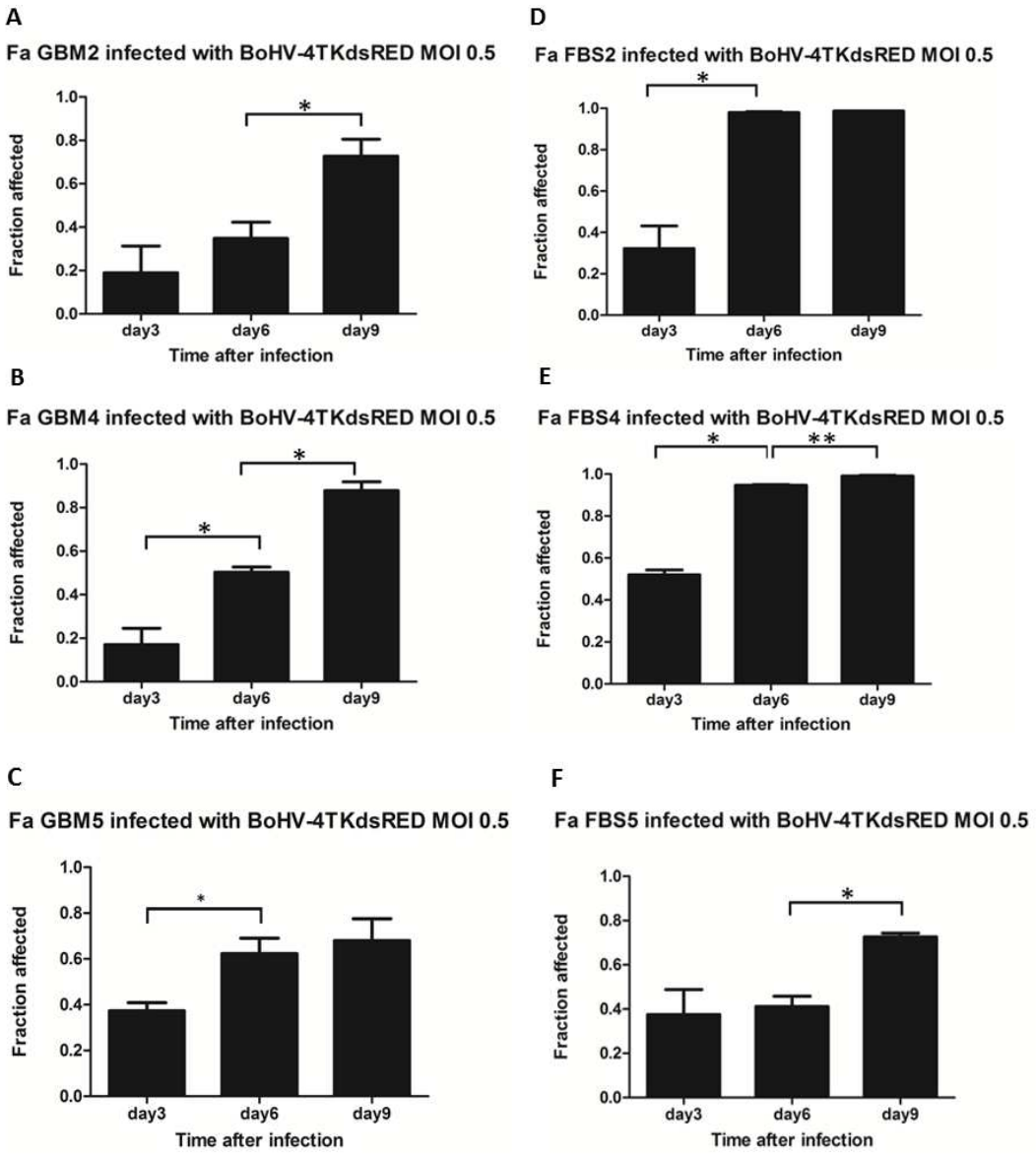

Figure 9. Fraction affected of GBM2 (A), GBM4 (B), GBM5 (C), FBS2 (D), FBS4 (E) and FBS5 (F) at 3, 6 and 9 days after infection with BoHV-4TKdsRED at MOI 0.5. (*$p<0.05$; ** $p<0.001$; mean ± SD)

In order to compare the killing ability of BoHV-4TKdsRED with the well-known effect of HSV-1 wild type on GSCs on our GSCs cultures, GBM2, GBM4 and GBM5 cells were treated with HSV-1 wild type at the same MOI 0.5 used for BoHV-4TKdsRED.

As expected GSCs were more sensitive to HSV-1 wild type infection than to BoHV-4TKdsRED infection. 9 days after infection GSCs were almost completely killed by the virus (Figure 10). Significant differences have been calculated between day 3-day 6 in each culture (p=0.0343 in GBM2, p=0.0034 in GBM4 and p=0.0191 in GBM5 respectively; unpaired t-test; n=3).

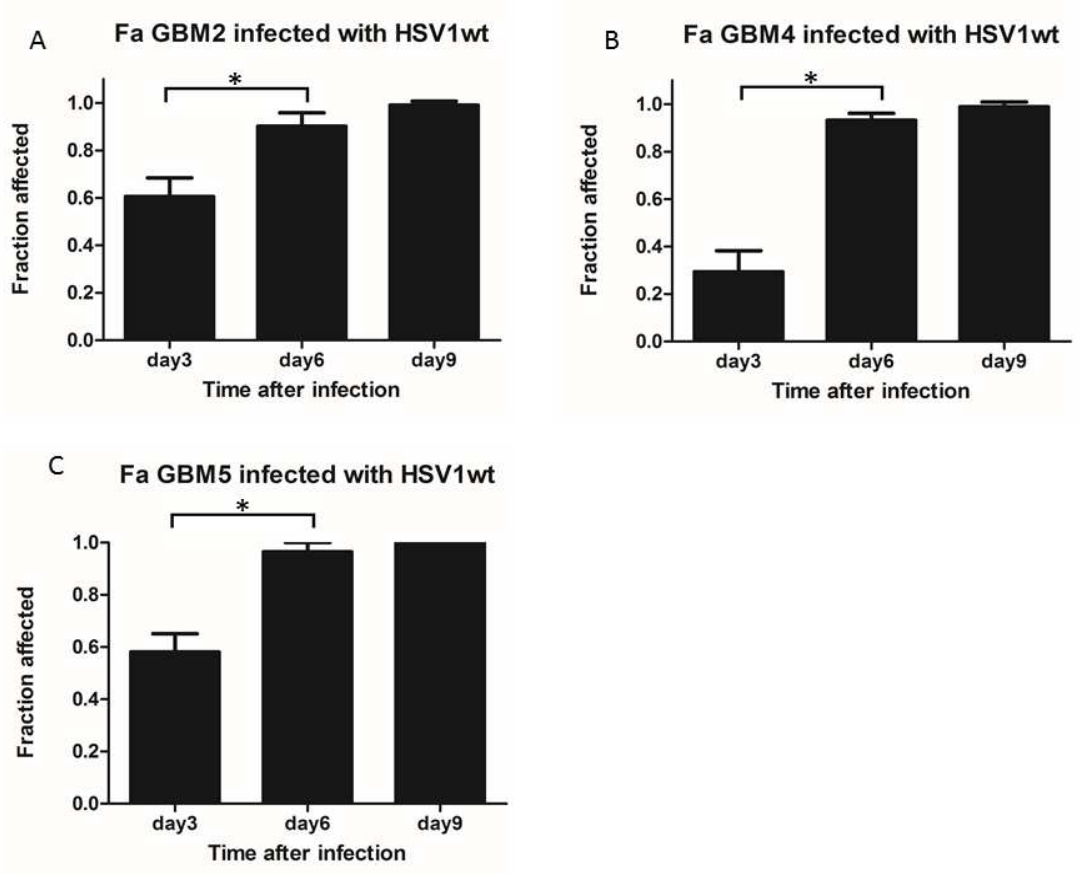

Figure 10. Fraction affected of GBM2 (A), GBM4 (B) and GBM5 (C) at 3, 6 and 9 days after infection with HSV-1 wild-type (HSV1wt) at MOI 0.5. (*p<0.05; mean ± SD)

5.2.4 Suicide Gene Therapy Protocol In GSCs And FBS Cultures

BoHV-4TKdsRED has been engineered to express HSV-1 TK, in order to make the infected cells sensitive to GCV (Redaelli *et al,* 2012). To evaluate the cytotoxic effect of the drug, different concentrations of GCV (10, 50 and 100 μg/ml) have been tested in our GSCs cultures. GBM2, GBM4 and GBM5 cells were plated at a density of $2*10^4$ cells/well and the day after treated or not with GCV. Cells were then counted after 3, 6 and 9 days and the fraction affected calculated as previously described.

In GBM2 cells GCV had a time- and dose-dependent increasing cytotoxicity (Figure 11A). Significant differences have been calculated between GCV 10 μg/ml-GCV 100 μg/ml and GCV 50 μg/ml-GCV 100 μg/ml at day 9 (p=0.02951 and p=0.0485 respectively; unpaired t-test; n=3).

The same effect has been observed in GBM4 cells (Figure 11B), with significant differences at day 3 between GCV 10 μg/ml-GCV 50 μg/ml (p=0.0152; unpaired t-test) and at day 9 between GCV 10 μg/ml-GCV 100 μg/ml (p=0.0023; unpaired t-test; n=3).

In GBM5 cells this effect could not be seen (Figure 11C). Significant differences have been calculated at day 3 between GCV 10 μg/ml-GCV 50 μg/ml (p=0.0377; unpaired t-test) and at day 9 GCV 10 μg/ml-GCV 100 μg/ml (p=0.0023; unpaired t-test; n=3), but the fraction affected remained almost unchanged between the different time points considered. This result can be due to a higher percentage of self-renewing cells in GBM5.

To test the combined treatment of virus and prodrug, GBM2, GBM4 and GBM5 cells were plated as already described, the day after infected with mock or BoHV-4TKdsRED at MOI 0.5 and after 24 hours treated using the range of GCV concentrations tested. The cell fraction affected has been calculated as already described.

The use of GCV increases the killing effect of BoHV-4TKdsRED in a time- and dose-dependent manner in each culture examined.

In GBM2, at day 3 and at day 6, the fraction affected of cells treated only with BoHV-4TKdsRED was significantly lower compared to the fraction affected with BoHV-4TKdsRED plus GCV 50 μg/ml and GCV 100 μg/ml (at day 3 p=0.034 and p=0.037 respectively; at day 6 p=0.037 and p=0.008 respectively; unpaired t-test; n=6) (Figure 11D).

In GBM4 cells a significant difference has been calculated at day 3 between cells treated with the virus alone and cells treated with the virus and GCV 50 μg/ml (p=0.037; unpaired t-test; n=3) (Figure 11E).

Also in GBM5 cells the combined treatment increased the fraction affected at each time point compared to the fraction affected with the virus alone (Figure 11F). Significant differences have been calculated between each condition at each time point examined (at day 3 BoHV-4TKdsRED-BoHV-4TKdsRED+GCV 10 μg/ml p=0.006, BoHV-4TKdsRED-BoHV-4TKdsRED+GCV 50 μg/ml p=0.0006, BoHV-4TKdsRED-BoHV-4TKdsRED+GCV 100 μg/ml p=0.0002; at day 6 BoHV-4TKdsRED-BoHV-4TKdsRED+GCV 10 μg/ml p=0.001, BoHV-4TKdsRED-BoHV-4TKdsRED+GCV 50 μg/ml p=0.0001, BoHV-4TKdsRED-BoHV-4TKdsRED+GCV

100 µg/ml p<0.0001; at day 9 BoHV-4TKdsRED-BoHV-4TKdsRED+GCV 10 µg/ml p=0.0005, BoHV-4TKdsRED-BoHV-4TKdsRED+GCV 50 µg/ml p<0.0001, BoHV-4TKdsRED-BoHV-4TKdsRED+GCV 100 µg/ml p<0.0001; n=6).

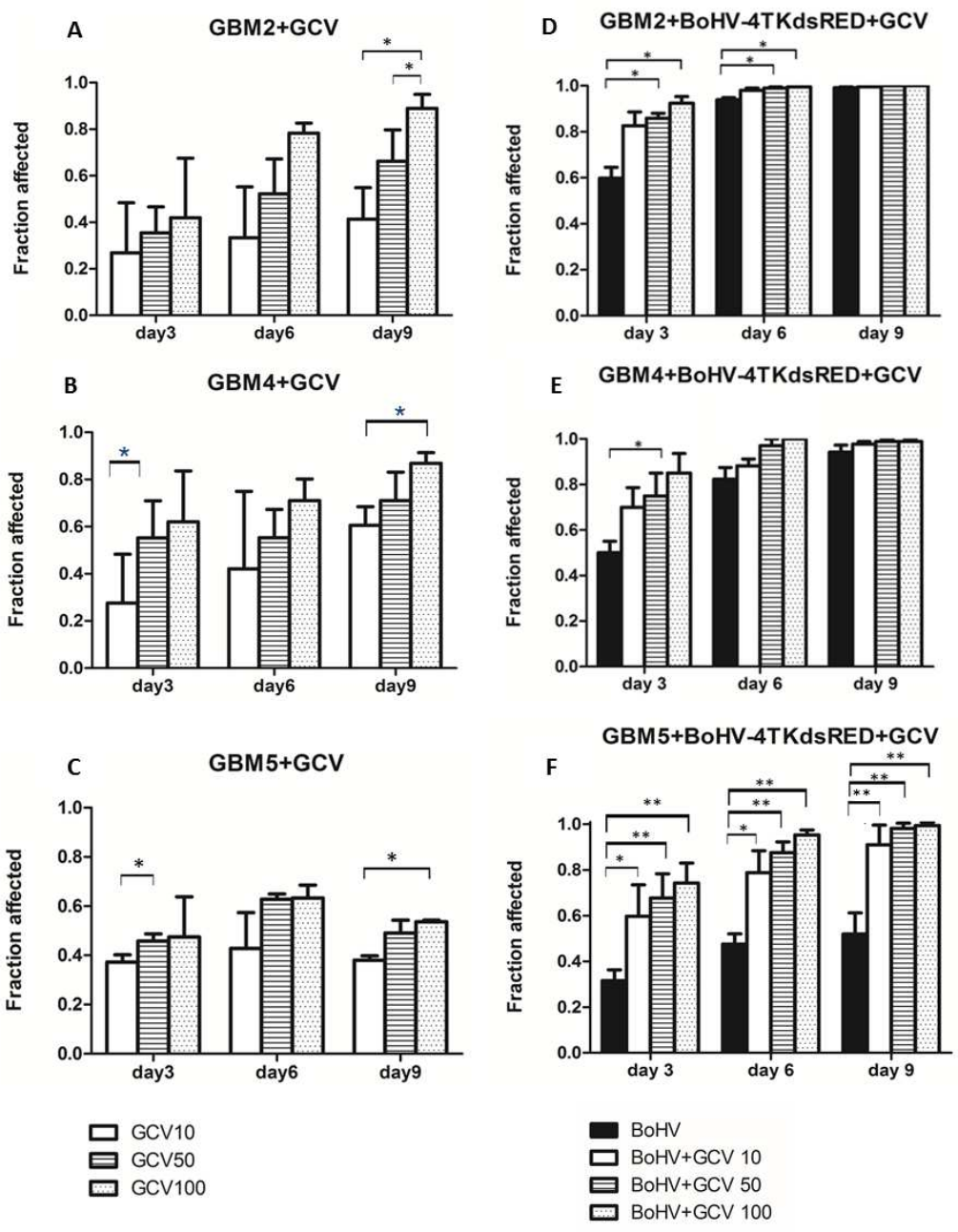

Figure 11. Fraction affected of GBM2 (A, D), GBM4 (B, E) and GBM5 (C, F) at 3, 6 and 9 days after treatment with BoHV-4TKdsRED (BoHV) at MOI 0.5 and/or GCV 10, 50 or 100 µg/ml (GCV 10, GCV 50, GCV 100). (*p<0.05; ** p<0.001; mean ± SD)

5.2.5 BoHV-4TKdsRED Infection Effect In GSCs Cultures: Autophagy

To understand the killing mechanism of BoHV-4TKdsRED in GSCs, a preliminary experiment with GBM2 cells was performed. GBM2 cells were infected with mock or BoHV-4TKdsRED at MOI 1 and 24 hours later treated with GCV 50 µg/ml or with PBS as a control for 24 hours before protein extraction. Nestin, Sox2 and GFAP expression have been checked to verify any possible variation after infection and/or GCV treatment. A very slight decrease in Nestin expression has been observed after BoHV-4TKdsRED+GCV treatment, while any other treatment seems to interfere with other markers expression. Two autophagy markers were tested: p62, an ubiquitin-binding protein involved in many cellular processes, which expression decreases under autophagy due to protein degradation (Bjorkoy *et al,* 2006; Moscat *et al,* 2012); LC3 (microtubule-associated protein light chain 3), an essential protein for autophagosome formation (Tanida *et al,* 2004). LC3 exists in two isoforms: LC3-I, cytoplasmic, and LC3-II, associated with the autophagosome membrane and increased during autophagosome formation (Kabeya *et al,* 2000).

Neither considered markers seem to be affected by any treatment (Figure12).

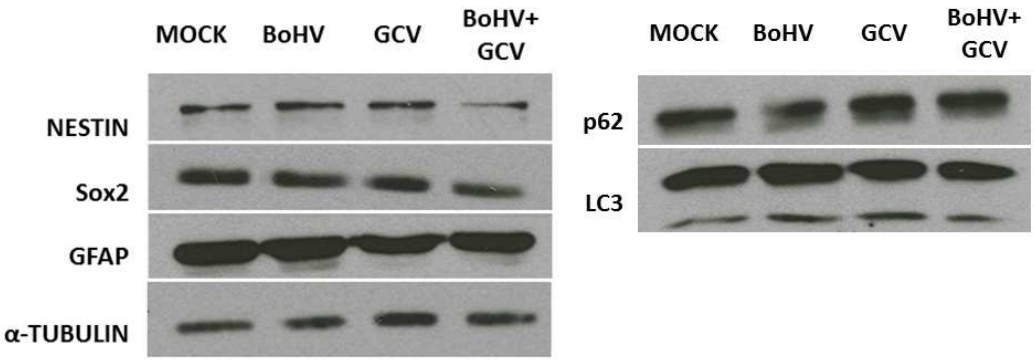

Figure 12. Western Blots performed on GBM2 treated or untreated cells with BoHV-4TKdsRED (BoHV) for 48 hours and GCV 50µg/ml for 24 hours. In the right panel α-tubulin was not detectable after stripping.

To further understand the effect of BoHV-4TKdsRED and/or GCV on GSCs, $5*10^5$ cells/well GBM2 and GBM5 cells were plated and treated as already described and with Rapamycin 1 µM, an autophagy inducer. Proteins were extracted in a wider range of time points. In GBM2 cells an LC3-II expression increase was seen in cells treated with GCV for 24 hours and in cells treated with BoHV-4TKdsRED for 72 hours plus GCV for 48 hours, but p62 expression was

not decreased. This could be a signal of autophagosome formation, not sustained with a complete activation of autophagy within the time points considered (Figure 13A).

In GBM5 cells the increase of LC3-II expression appeared in cells treated with GCV for 48 hours and in cells treated with BoHV-4TKdsRED for 72 hours plus GCV for 48 hours, without a decrease of p62 expression. Also in this case it is possible to presume an initial autophagosome formation within the time points considered (Figure 13B).

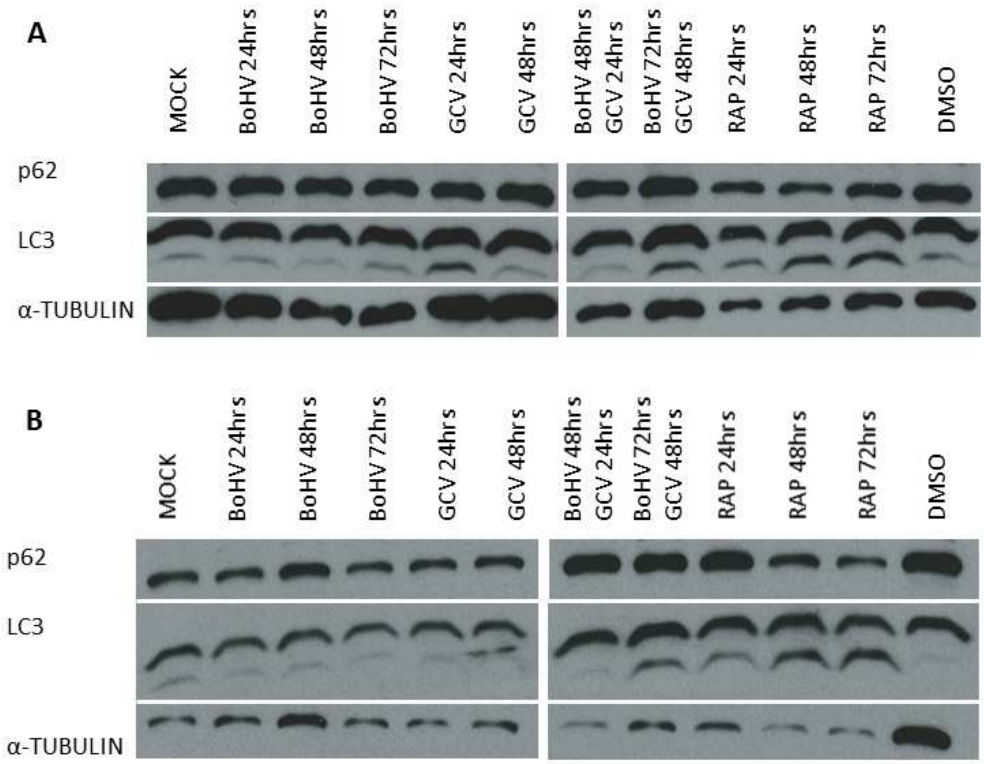

Figure 13. Western Blots performed on GBM2 (A) and GBM5 (B) treated or untreated cells with BoHV-4TKdsRED MOI 1 (BoHV), GCV 50 µg/ml (GCV), Rapamycin 1 µM (RAP) and DMSO.

5.2.6 BoHV-4TKdsRED Infection Effect In GSCs Cultures: Apoptosis

The possible role of BoHV-4TKdsRED in apoptosis activation in GSCs has been investigated. GBM2 and GBM5 cells were plated at a density of $2*10^4$cells/well, the day after infected with mock or BoHV-4TKdsRED MOI 1 or with Paclitaxel 100 nM, an apoptosis inducer. After 24 hours cells were treated or not with GCV 50 µg/ml. After preliminary experiments, cells were analyzed 72 hours after BoHV-4TKdsRED infection for AnnexinV/PI fluorescence on BD FACScalibur™. In both GSCs cultures was detected a shift in the Annexin V fluorescence peak, compared to the unlabeled control, also in cells mock treated. This made it impossible

to correctly determine fluorescence changes and so whether apoptosis is activated or not after treatments (Figure 14).

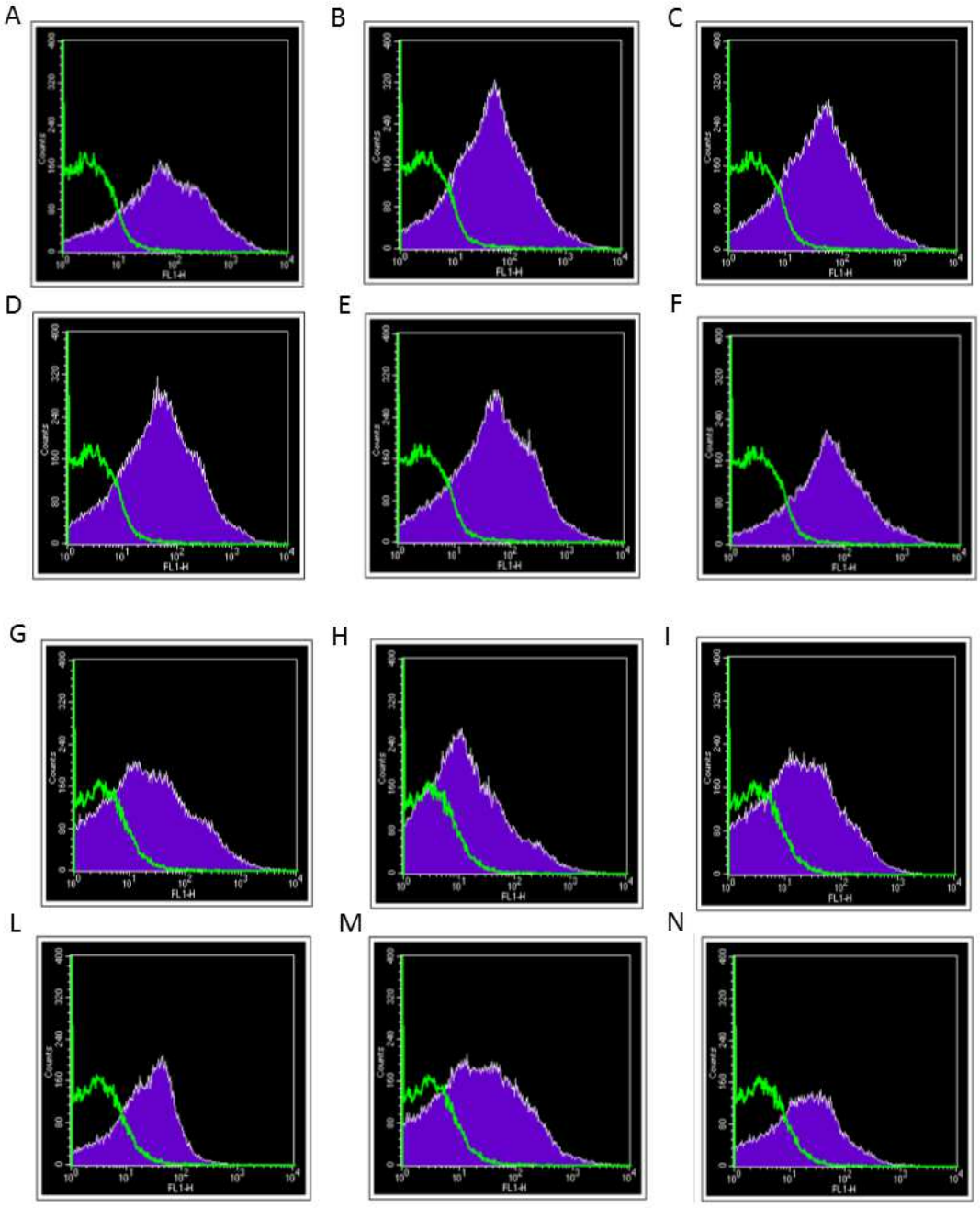

Figure 14. Annexin V fluorescence on GBM2 cells (A MOCK, B BoHV-4TKdsRED MOI 1, C GCV 50 µg/ml, D BoHV-4TKdsRED+GCV, E Paclitaxel 100 nM, F DMSO) and GBM5 cells (G MOCK, H BoHV-4TKdsRED MOI 1, I GCV 50 µg/ml, L BoHV-4TKdsRED+GCV, M Paclitaxel 100 nM, N DMSO). Green line: fluorescence of unlabeled control; Purple: fluorescence of sample.

To verify whether this shift is linked to some peculiar cell characteristics, the same experiment with FBS5 cells has been performed. In this case it was possible to discriminate

the negative peak from the positive one in the AnnexinV fluorescence and it was observed an apoptosis induction after BoHV-4TKdsRED infection and after the combined treatment (Figure 15).

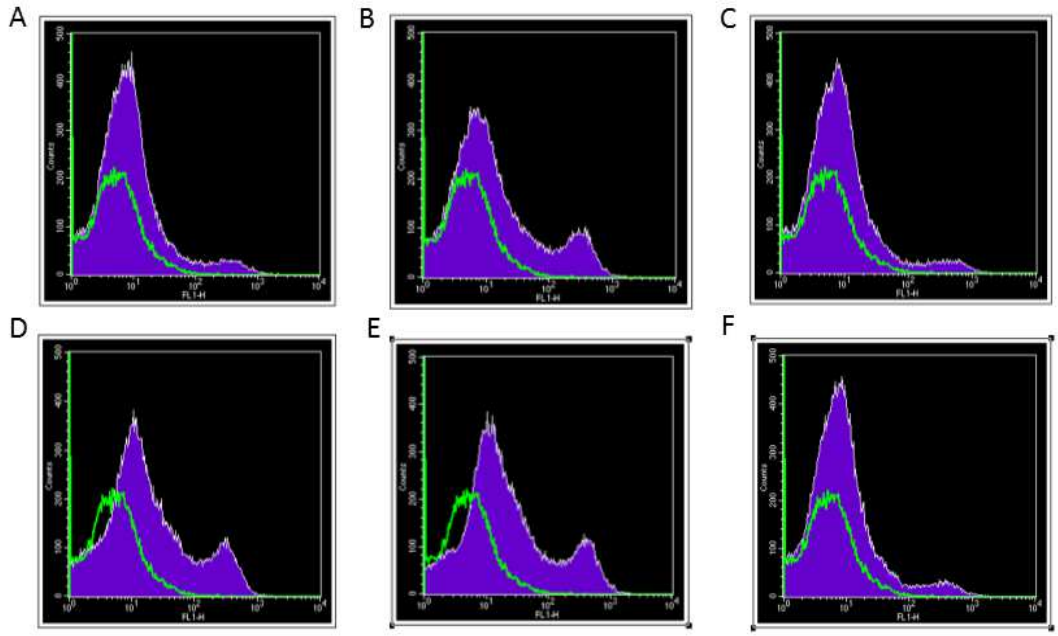

Figure 15. Annexin V fluorescence on FBS5 cells (A MOCK, B BoHV-4TKdsRED MOI 1, C GCV 50 µg/ml, D BoHV-4TKdsRED+GCV, E Paclitaxel 100 nM, F DMSO). Green line: fluorescence of unlabeled control; Purple: fluorescence of sample.

5.2.7 BoHV-4TKdsRED Infection Of Self-Renewing Spheres

In order to verify whether BoHV-4 is able to infect self-renewing cells, $2*10^4$ GBM5 cells were seeded and the day after infected at MOI 0.2 with BoHV-4EGFPΔTK (Donofrio *et al,* 2002), which allows a better visualization of reporter protein expression. Cells were monitored and after 7 days where dissociated and plated at clonogenic density (1 cell/well). Cell were monitored until spheres formation. Five spheres have been identified. BoHV-4EGFPΔTK is able to infect self-renewing cells without causing cytopathic effect (Figure 16).

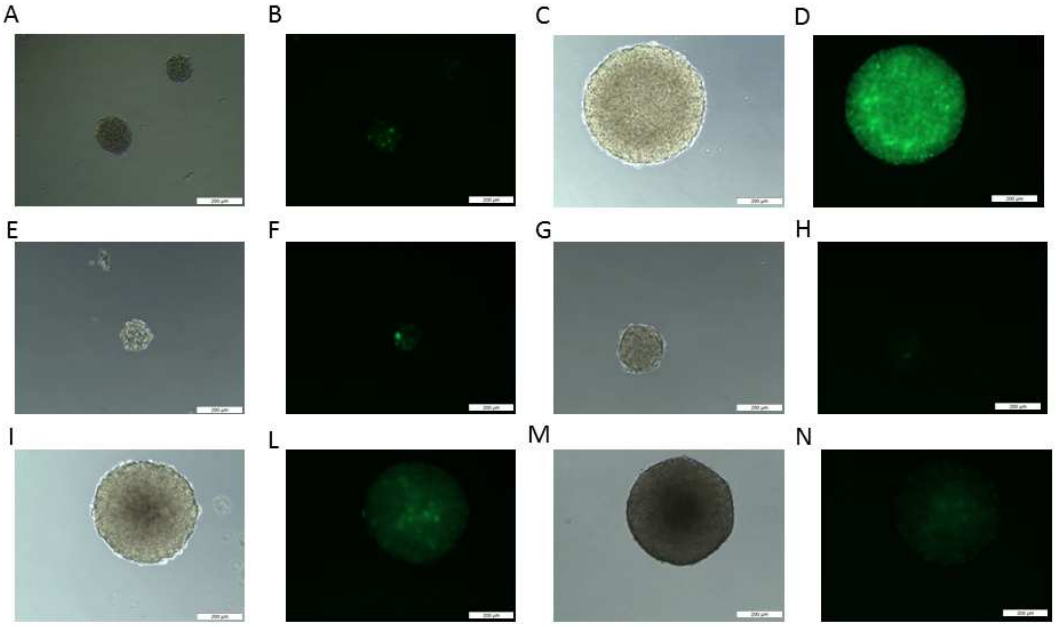

Figure 16. (A, B) GBM5 cells 6 days after BoHV-4EGFPΔTK infection; (C-N) Spheres formed in a clonogenic density (1 cell/well). 10X; scale bar 200 µM.

Spheres were mechanically dissociated and the infection was monitored through EGFP expression until 60 days after infection. Also in sphere-derived cultures EGFP expression was observed without cytopathic effects (Figure 17).

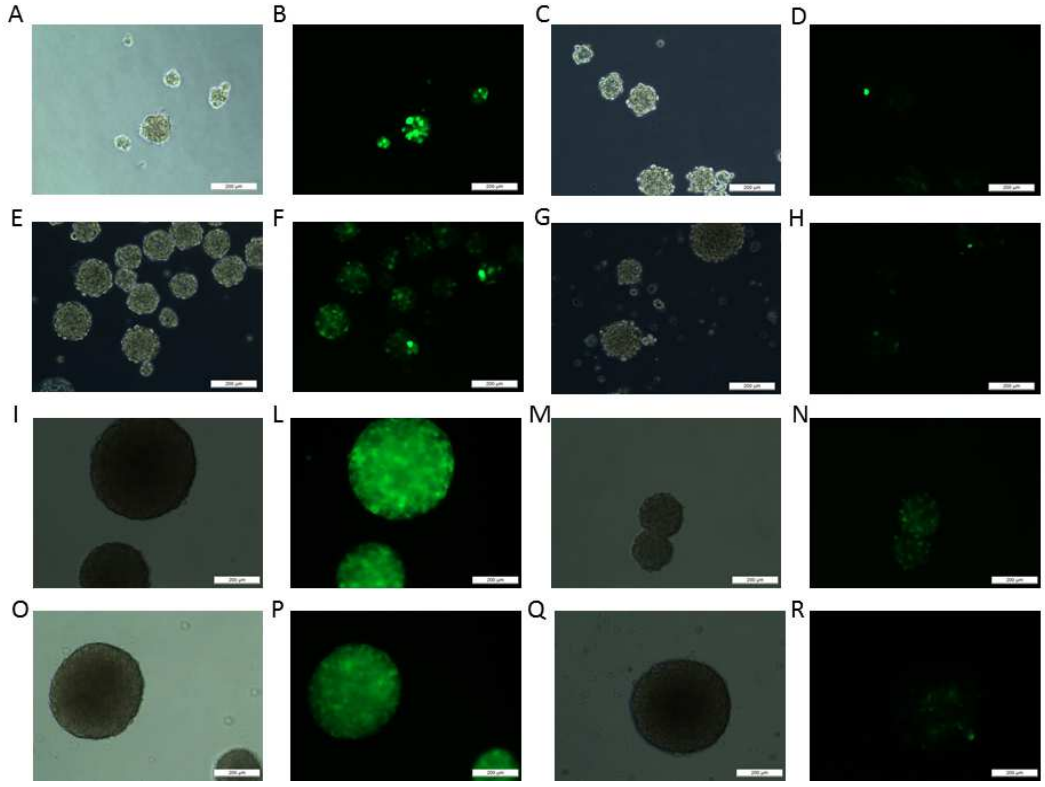

Figure 17. (A-H) GBM5 spheres after mechanical dissociation; (I-R) GBM5 spheres 60 days after infection. 10X; scale bar 200 µM.

The same experiment was performed with GBM2 cells, but in this case CPE was seen within the first 7 days of infection and no spheres were obtained after dissociation (Figure 18).

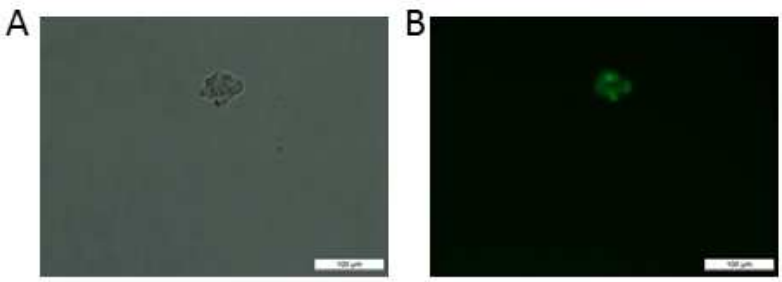

Figure 18. (A, B) GBM2 cells 6 days after BoHV-4EGFPΔTK infection. 20X; scale bar 100 µM.

6. DISCUSSION

GBM is the most malignant and most frequent primary brain tumor in adults and it is still incurable, despite great progresses in today therapies (Stupp *et al,* 2009). In recent years the resistance of this tumor to therapies has been ascribed to the existence of some cells in the tumor mass with stem characteristics (Singh *et al,* 2003; Galli *et al,* 2004; Bao *et al,* 2006). Glioma Stem Cells are a low replicating, chemo- and radio-resistant subpopulation of cancer cells, so the goal of GBM therapies is the specific targeting of these cells (Chen *et al,* 2012). In order to selectively target GSCs novel therapeutic strategies have been studied and, among these, Gene Therapy using viral vectors can be a promising approach (Dey *et al,* 2010). In the present study a BoHV-4-based vector engineered to express HSV-1 TK and the Red Fluorescent Protein as a reporter gene has been used. The presence of HSV-1 TK enables the vector to convert the prodrug GCV in a toxic metabolite in infected cells. This vector has already demonstrated its interesting role in infecting and killing immortalized glioma cell lines of different species and a primary human GBM culture (Redaelli *et al,* 2012). In the present study it has been used for the first time against GSCs cultures.

Following published protocols(Wakimoto *et al,* 2009), GSCs cultures (GBM2, GBM4 and GBM5) were established from fresh human glioblastoma samples. From the same specimens, primary adherent cultures (FBS2, FBS4 and FBS5) were also obtained. The stem characteristics of our GSCs was verified through self-renewal assays and stem markers expression. Our GSCs cultures demonstrated different percentages of sphere-forming cells, when plated at clonogenic density. The cell growth of our culture has been studied and GSCs showed a gradual growth, whether serum-cultured cells, in particular FBS4 and FBS5, have a faster growth than their stem counterparts. This is in line with data reported in literature about the low replication rate of GSCs (Bao *et al,* 2006).

The Bovine Herpesvirus vector BoHV-4TKdsRED is able to efficiently infect GSCs and serum-cultured cells; it is able to slightly replicate in GBM2, GBM4, FBS2 and FBS4 cells within 72 hours. In GBM5 cells viral replication was slower and in FBS5 cells, even after 144 hours of infection the viral titer didn’t get over the initial input; this probably because cells died before the virus completed the replication cycle. The viral replication was not high in GSCs, probably due to the slow replication rate of the cells. Indeed, the virus uses the cell machinery to replicate itself and so it is dependent on the mitotic activity of the infected cells (Capocefalo *et al,* 2009).

Despite its low replication rate, BoHV-4TKdsRED performs a cytopathic effect and kills GSCs and FBS cells. The fraction affected after 9 days in GSCs varied from 67% to 87%, with GBM5 cells more resistant to infection than other cultures. FBS cells resulted more sensitive to infection than GSCs, in particular FBS2 and FBS4 cells were almost completely killed after 9 days. The different killing ability of BoHV-4TKdsRED in cells derived from different patients stresses the high heterogeneity of GBM.

The killing ability of BoHV-4TKdsRED in GSCs was lower than the one showed by HSV-1 wild type. Of course, it is important to keep in mind that HSV-1 wild type has a high neuropathogenicity, which is not the case of BoHV-4, and that the use of wild type HSV-1 is impossible in patients because it causes lethal encephalitis. Conversely, BoHV-4 resulted no neuropathogenic in animal models and would be a safe viral vector (Donofrio *et al,* 2006).

Experiments of Suicide Gene therapy highlighted a time- and dose-dependent increase of BoHV-4TKdsRED with a combined used of different doses of the prodrug GCV in GSCs. Also in this case GBM5 cells resulted more resistant to treatments. This resistance could derive from the higher percentage of self-renewing cells in GBM5 culture.

The further question was about how BoHV-4TKdsRED exerts its cytopathic effect. Autophagy is a mechanism involved in many cell functions and can have a pro-survival or a pro-death role (Ouyang *et al,* 2012). Its role in cancer is still not completely understood and an autophagy downregulation seems to be correlated with tumor progression in GBM (Huang *et al,* 2010). BoHV-4TKdsRED infection alone is not able to induce autophagy, but the combined treatment with GCV induces a first autophagosome formation after 48 hours, as detected with LC3-II increased expression in Western Blots, in GBM2 and GBM5 cells. These results were not sustained by a p62 expression decrease, suggesting an incomplete autophagy activation. Whether this incomplete activation is due to a downregulated ability of GSCs to perform autophagy or to a non sufficient exposition time of the cells to the treatments, is a point that is still unclarified.

Apoptosis is the most known type of Programmed Cell Death and is downregulated in cancer cells (Krakstad *et al,* 2010). BoHV-4TKdsRED resulted able to induce apoptosis in immortalized glioma cell lines and in a primary human GBM culture, in particular when used in combination with GCV (Redaelli *et al,* 2012). The apoptosis activation was investigated also in GSCs through Annexin V/PI FACS analysis. Annexin V fluorescence was not reliable in GBM2 and GBM5 cells. So it was not possible to determine whether the virus alone or the

combined treatment induce apoptosis in GSCs. The same result was not observed in FBS5 cells, where an increase in Annexin V fluorescence was detected in cells treated with BoHV-4TKdsRED and with the combined treatment. Probably the unusual Annexin V fluorescence in GSCs is due to an unspecific binding of the die. Annexin V binds to Phosphatidylserine, which is exposed on the cell membrane during apoptosis (Demchenko 2012). However, this method has some pitfalls and can determine false positives (Demchenko 2013). The reason of the Annexin V fluorescence peak shift detected in our GSCs is still unknown.

The real goal of Gene Therapy is the selective killing of GSCs, in particular of self-renewing cells. Another BoHV-4-derived vector, BoHV-4EGFPΔTK was used to verify the infection of self-renewing cells. BoHV-4EGFPΔTK has the same characteristics of BoHV-4TKdsRED, except for the lack of HSV-1 TK and the EGFP in place of RFP as reporter gene. EGFP allows a better visualization of infected cells than RFP. The self-renewal assay performed after GBM5 infection with BoHV-4EGFPΔTK showed that the vector is able to infect self-renewing cells, but didn't exert a cytopathic effect in these cells. Even 60 days after infection and after mechanical dissociation of the spheres, cells appeared healthy.

For the first time this study demonstrated the ability of BoHV-4TKdsRED to infect human GSCs. From the results obtained so far, we can consider BoHV-4TKdsRED a good candidate for Gene Therapy of GSCs, but further analysis should be performed to verify the killing mechanisms involved. Moreover, further studies are necessary to assess whether the combined treatment of BoHV-4TKdsRED and GCV is able to kill also self-renewing and not only the rapidly-proliferating compartment of GSCs.

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