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Use of miRNA target sequences to control essential genes of HSV1-based oncolytic vectors.

Settore Scientifico Disciplinare MED/07

Dottoranda
Dott.ssa Maria Giovanna Foschini

Primo Tutore
Dott.ssa Peggy Marconi

Secondo Tutore
Dott. Rubén Hernández-Alcoceba

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Introduction

1. Concept of Gene Therapy

Gene therapy is a form of molecular medicine that promises to provide new treatment for a large number of inherited and acquired human diseases when and where conventional clinical procedure are less effective. These diseases include monogenic disorders, such as cystic fibrosis, but also more complex disorders, such as cardiovascular disease, disease of nervous system, autoimmune disease and cancer.

The basic concept of gene therapy is simple: introduce into target cells a piece of genetic material that will result in either a cure for the disease or slowdown in the progression of it.

In the case of genetic diseases caused by a mutation in a specific gene, gene therapy usually involves the delivery of a functional copy of alterated gene into a target cell or tissue to achieve a therapeutic benefit [1]. Furthermore, it can also be a tool for the treatment of non-genetic and polygenic disorders such as cancer by delivering genes that stimulate immune response, suicidal genes inducing cell death, genes modifying cellular information or developmental program, or genes producing a therapeutic protein with specific functions [2].

Until June 2011, the 81,4% of gene therapy clinical trials has address cancer (64,7% of all gene therapy trials), vascular disease and inherited monogenic diseases (Fig.1). The gene therapy application in the first two have a higher prevalence because of their enormous impact and potentially fatal outcomes, the latter because the replacing a well-defined defective gene with its correctly functioning counterpart has an obvious appeal [3,4].

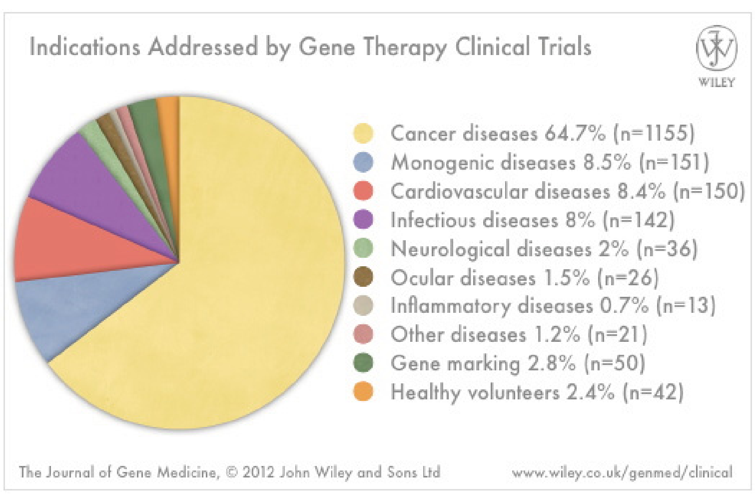


Figure 1. Clinical trials based on gene therapy from 1989 until July 2011 [5].

1.1. Gene transfer Therapy

The simplest way of gene delivery is injecting naked DNA encoding the therapeutic protein, but because of low efficiency there is a need to use special molecules and methods to improve gene delivery. A vector can be described as a system fulfilling several functions, including: enabling delivery of genes into the target cells and their nucleus; providing protection from gene degradation and ensuring gene transcription in the cells. The administration of gene therapy vectors requires that they should not only be targeted and safe, but also protected from degradation, sequestration or immune attack.

There are two kind of vectors for gene transfer therapy: viral vectors, such as retroviral, adenoviral, adeno-associated viral and herpes simplex viral vector, and non-viral vectors for gene transfection, as plasmids, liposomes and nanoparticles. Each type of vector presents its own vantages and disadvantages.

Regarding these two types of used vectors, differences are observed from 1989 to 2011, although the viral vectors remain the most used (Fig.2). The adenoviral vectors are the most used since 2004, occupying about one-third of clinical trials there is an increased in the last years in the use of some viral vectors such as adeno-associated vectors (AAVs) and lentiviral vectors. (Fig.2). The herpes simplex virus based vectors are use slightly less than the past, but the clinical trials based on it are in phase III or already successfully concluded.

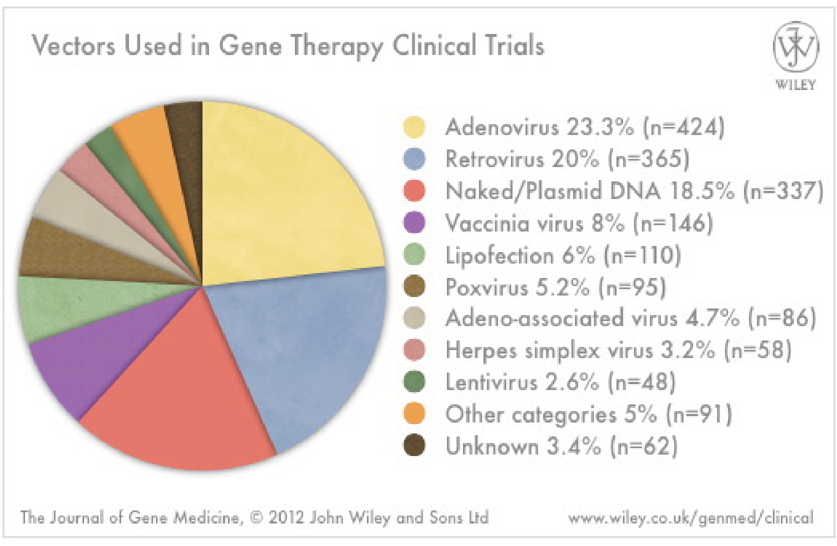


Figure 2. Gene Therapy vectors used in clinical trials [5].

1.2. Viral vectors

The basic concept of viral vectors is to harness the innate ability of viruses to deliver genetic material into the infected cell. Different viruses are studied and currently used as vectors: derivate from oncoretrovirus, lentivirus, adenovirus, adeno-associated virus (AAV) and herpes simplex virus (HSV). Each of these vectors is characterized by a set of different properties that make it suitable for some applications and unsuitable for others. The five main classes of viral vectors can be categorized in two groups according to whether their genomes integrate into host cellular chromatin (oncoretroviruses and lentiviruses) or persist in the cell nucleus predominantly as extrachromosomal episomes (AAVs, adenoviruses and herpes simplex viruses). Non-integrating vectors can mediate persist transgene expression in non-proliferating cells, while integrating vectors are the choice if stable genetic alteration needs to be maintained in dividing cells. However, the integration is not a guarantee of stable transgene expression from integrated vector genomes since transcription can be gradually silenced over time [6].

Oncoretrovirus vectors were the first class of viral vector to be developed and have been the most widely used in clinical trials. They have traditionally been the vectors of choice for the *ex vivo* transduction of repopulating hematopoietic stem cells; the limitation of these vectors is that they can only transduce dividing cells. To overcome this limitation, the attention have been redirect to the use of lentiviral vectors that have the capacity to naturally translocate into the nucleus through the intact nuclear membrane and transduce non-dividing and dividing cells. Lentiviral vectors seem to be very promising tool for the treatment of different gene therapy disorders.

Recombinant AAV vectors are one of the most encouraging vector systems for safe long-term gene transfer and expression in non-proliferating tissues. The small size and simplicity of the AAV vector particles makes possible to administer high dose of the viral vector systemically without eliciting acute inflammatory responses or toxic effects, the major limitation is the small cloning capacity.

Adenovirus vectors are, arguably, the most efficient class of vector in terms of efficiently tissues transduction and delivering genetic cargo to the cell nucleus [7]. Major changes have been done to AV vectors in order to reduce toxicity and immunogenicity and the development of helper-dependent adenoviruses (HD-Ads), that are deleted for all viral genes, has been the most important vector modification to decrease immunogenicity,

prolong transgene expression and improve the prospect of adenovirus vectors for long-term gene therapy [8].

Herpes simplex virus type 1, whose characteristics and applications will be fully described later, is the largest and most complex of all the viruses that are being developed for gene therapy, and one important feature of this vector is its capacity to carry large fragments of foreign DNA. In fact, in specific therapeutic applications, the space availability for the incorporation of exogenous DNA is another criterion that influences the choice of the viral vector.

Despite some limitations on the use of viral vectors regarding safety and reproducibility, actually they are the most used gene transfer vehicles [9].

1.3. Viral vectors for cancer

A number of new therapies have been developed for treatment of cancer, and the knowledge of the basic defects that occur in malignant tumors has lead to the conclusion that is essential the association of different therapeutic approaches to eradicate these malignancies [10].

The interactions between tumor cells and their microenvironment have been shown to play a very significant role in the initiation, progression and therapeutic resistance of cancer. In fact the tumor microenvironment is constituted of non-transformed host stromal cells such as endothelial cells, fibroblasts, various immune cells, and a complex extracellular matrix (ECM) secreted by both the normal and neoplastic cells embedded in it. These tumor-stromal interactions are capable of altering the delivery and effectiveness of therapeutics into the tumor and, in particular, the tumor microenvironment plays an important role in restricting viral spread and promoting tumor growth [11, 12].

Several different strategies have been used in an array of different tumor types, such as lung, stomach, skin, ovarian and CNS cancers.

There are three systems for cancer gene therapy and virotherapy: (i) oncolytic virotherapy, (ii) cancer-specific promoter systems and (iii) tissue-specific promoter systems.

Oncolytic viruses (OV) are emerging as promising options for treatment of cancer, in particular when they are used in combination with standard therapies or if they are adapted as vectors to deliver therapeutic genes [13]. Replication-competent oncolytic viruses display the unique property of selectively killing cancer cells via their normal replication pathways, construction of oncolytic viruses that retain their ability to replicate,

with a subsequent release of progeny that are similarly able to spread throughout the tumor and infect other cancer cells without toxicity for neighbouring healthy cells, has become a major area of therapeutic cancer research. These viruses, in addition to directly killing cancer cells by virtue of the virus life cycle, can be used as viral vectors to deliver transgenes, whose expression will enhance their inherent anti-tumour activity. This can be achieved through (a) the expression of potentially cytotoxic enzymes, (b) the induction or enhancement of a tumour-specific immune response, (c) the inhibition of angiogenesis [14], (d) or through the use of specific promoters. A number of oncolytic viruses have already moved to the clinic as anticancer agents. Recent trials using genetically engineered viral strains, such as adenovirus and herpes simplex virus, have been encouraging, showing that these viruses are relatively non-toxic for normal cells, while remaining lytic in tumor cells. However the targeting of tumor cells has not been absolute and it may be possible to improve tumor specificity by retargeting infection to tumor cell surface receptors or by targeting viral replication to specific types of cells or cells that are presenting a peculiar condition.

HSV-1 vectors have many advantages over other oncolytic viruses owed to several unique features of the HSV-1 life cycle. First, HSV-1 is a well-studied large DNA virus with a genome of 153 kbp, encoding at least 89 proteins, with unique genetic flexibility. Many viral proteins are non-essential for the multiplication of the virus in cultured cells, therefore permitting to replace more than 40 kbp of the virus genome with foreign DNA, yet still leave the virus to replicate in the appropriate cellular environment. Furthermore, multiple genes associated with virulence can be deleted or modified without affecting the virus capacity to replicate within and destroy tumour cells during its lytic phase. This unique property of HSV-1 is critical for cancer therapy, as the potency of most oncolytic viruses needs to be enhanced by the expression of additional transgenic anticancer functions. Second, HSV-1 possesses powerful inherent cytolytic potential for most types of cells, including murine cells, thus allowing evaluating both the toxicity and safety of HSV-1 vectors in murine syngenic cancer models. Third, anti-herpetic drugs (acyclovir, forscanet) are available and provide a safety mechanism in case undesired local or systemic infection occurs. Finally, HSV does not integrate into the cellular genome and remains in an episomal state, so it cannot cause insertional mutagenesis.

2. Basic biology of Herpes simplex virus

2.1 Virus structure

Herpes simplex virus type 1 (HSV-1) is a human pathogen belonging to the alpha-*Herpesviridae* subfamily of herpesviruses. It is an enveloped, double-stranded (ds) DNA virus. The mature virus particles, 120-300 nm size, consist of different components: external envelope, tegument, icosahedral capsid and viral genome [15].

The envelope contains a host-cell derived trilaminar lipid layer in which are embedded 13 different glycoproteins responsible for host cell recognition and entry [15, 16].

The tegument is an amorphous layer, containing at least 20 different proteins with structural and regulatory roles at different stages of the virus life cycle. Its contains proteins such as virus protein 16 (VP16), VP22, virus shut-off (*vhs*) proteins that are collectively important for viral gene expression [15, 16, 17], degradation of host cell mRNA [18, 19, 20], viral particle assembly and inhibition of innate immune responses that can repress virus gene expression [21].

The icosahedral capsid is composed of 162 capsomers, a multiple structural proteins organized in 150 extravalent and 12 pentavalent, that encapsidate the viral genome [22, 23].

The HSV-1 genome consists of a 152 kb linear double stranded DNA arranged as long and short unique segments (U_L and U_S) each flanked by inverted repeated sequences (Fig.3). Unique region genes are present as single copy; those in the repeated segments are present in two copies. The HSV-1 DNA encodes about 89 unique transcriptional units; at least 84 of these transcriptional units encode proteins, divided in essential and non-essential functions. The majority of virus genes are contiguous without introns thus facilitating their manipulation [22, 23].

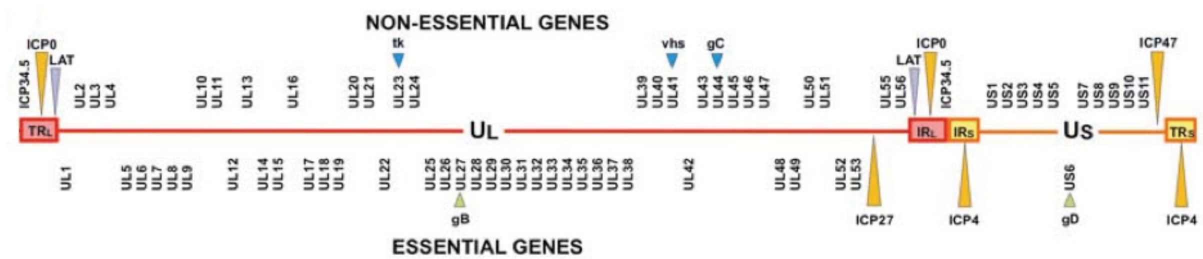


Figure 3. Schematic representation of HSV-1 genome.

HSV-1 gene nomenclature is based upon the position of the gene within the long and short segments. Genes within the long segments are designed as U_L1 to U_L56 and genes in the short segments are designed as U_S1 and U_S12. (Figs. 3 and 4).

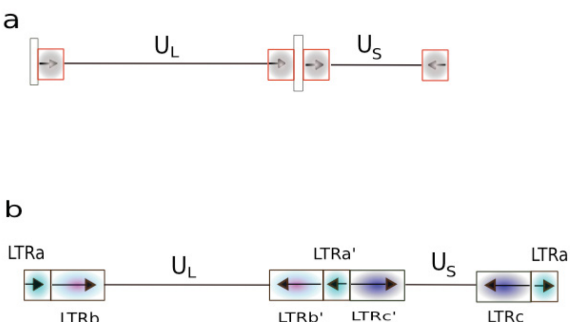


Figure 4. a) The genome of HSV-1 has a unique long (U_L) and a unique short (U_S) regions, flanked by inverted repeated sequences, named LTR a, b, c in diagram b. The number of copies of LTRs repeat may vary and the repeats allow rearrangements of the unique regions and HSV exists as a mixture of four isomers.

2.2 Viral infection

HSV-1 enters into the host cells by two different ways: endocytosis and fusion at the plasma membrane. The first way appears to be unique because it is likely not mediated by formation of clarthrin-coated pits. In another way the fusion is pH independent and requires the participation between multiple viral glycoproteins (gB, gC, gD, gH and gL) and cellular receptors (Fig. 5). This last mode of viral entry is initiated by interaction of viral gC and/or gB with heparan sulfate (HS), followed by interaction of gD with one of its three receptors. Entry of HSV into cells involves interactions between the viral receptor-binding protein gD and the gD receptors. These receptors include HVEM, a member of tumor necrosis factor-a (TNF a)/nerve growth factor (NGF) receptor family [24]; nectin-1 (CD111), a member of the IgG superfamily; nectin-2, and 3-O-sulfated HS. When gD binds to its receptors, there are conformational changes in gD which apparently activate gB and gH/gL, so that these glycoproteins promote fusion involving the virion envelope and cellular membranes.

It has been proposed a sequential model for fusion via HSV-1 glycoproteins whereby gD is required for Phase I, gH/gL is required for phase II, and gB is required for phase III. Other factors that may affect viral entry and/or intracellular signalling include: (1) the capability of gB to rapidly mobilize lipid rafts which might serve as a platform for entry and cell

signaling; (2) the release of plasma membrane Ca^{2+} stores and the increase in intracellular Ca^{2+} triggered by the engagement of nectin by gD and of integrin-αv subunits by gH, respectively.

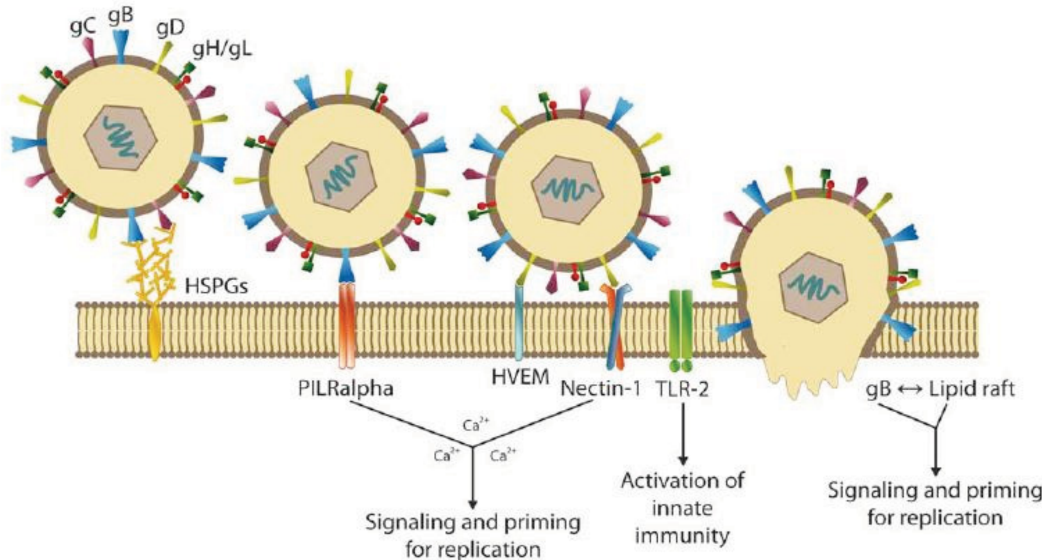


Figure 5: HSV-1 mechanism of entry into the host cell [10].

2.3 Gene regulation and viral particle assembly

After internalization, de-enveloped HSV particles travel to nucleus where the viral genes are expressed in a tightly regulated, interdependent temporal sequence [25, 26] and consist of immediate early/α (IE), early/β (E) and late/γ (L) genes.

The tegument protein VP16 is critical for rapid and high level expression of the IE genes. Upon envelope-plasmalemma fusion, VP16 enters the cytoplasm of the cell with the nucleocapsid, and it is transported to the host cell nucleus with the viral genome. In its role as an activator of IE transcription, VP16 forms a multi-component complex with at least two cellular proteins, Oct-1 and HCF, which is targeted to specific upstream TAATGARAT enhancer recognition sequences in IE promoters [27, 28].

The IE gene products (ICP0, ICP4, ICP22, ICP27, ICP47 and $U_S1.5$) induce expression of E genes that encode enzymes necessary for viral DNA replication, and L genes that express structural proteins that are assembled into new viral particles into the nucleus.

The E gene products (including viral DNA polymerase, single-stranded DNA binding protein, origin binding protein, DNA primase helicase) form complexes with the parental viral DNA, and carry out replication at one or several of the three HSV origins of DNA synthesis [29].

Concomitant with initiation of DNA synthesis, L genes are expressed. DNA concatamer progenies are cleaved into unit-length monomers and incorporated into capsids to form the nucleocapsids that bud through the nuclear membrane [10]. Capsids are assembled in the nucleus but the tegument and the envelope are acquired in the cytoplasm, most probably by budding into endosomes, and are released by exocytosis at cell membranes.
The virus replication cycle leads to rapid cell death and release of new viral particles during cell lysis.

2.4 Function and activities of ICP27 gene

ICP27 is a 63 kDa immediate early protein that localizes in the nucleus, is phosphorylated and its essential for viral replication. It regulates both cellular and viral gene expression through transcriptional and post-transcriptional activities [30].
At early times after infection, 3 hours, ICP27 mediates the inhibition of host cell splicing, which contributes to the shut off of host protein synthesis because cellular pre-mRNAs are not properly processed.
ICP27 interacts with a number of splicing factors, like serine/arginine-rich proteins (SR proteins) essentials for spliceosomal assembly. The activity of SR proteins is regulated by phosphorylation and dephosphorylation; ICP27 recruits a predominantly cytoplasmatic SR protein specific kinase (SRPK1) to the nucleus and this results in the aberrant phosphorylation of SR proteins [31], with consequent alteration of their functionality.
Also at early times after infection, about 4 hours, ICP27 interacts with cellular RNA polymerase II (RNAP II) and specifically with C-terminal domain (CTD) through both its N-terminal leucine-rich region and its C-terminal zing-finger domain [32]. ICP27, by interacting with RNAP II, recruits RNAP II to viral replication sites and consequently stimulates expression of viral DNA replication proteins. The mechanism by which ICP27, also, stimulates L gene expression involves mainly post-transcriptional activities. Post-transcriptional effects of ICP27 were initially inferred from its stimulation of reporter gene expression in transfected cells in which the stimulation appeared to be dependent on sequences in the 3'-untraslated region of the reporter gene [33]. Similarly, a late polyadenilation factor (LFP) was reported to be dependent on ICP27 [34].
ICP27 also recruits heat shock 70 kDa protein (Hsc70) to nuclear foci that remains at the periphery of the replication compartments [35].

These foci have been named Virus-Induced Chaperone-Enriched (VICE) domains because they contain Hsc70, other chaperone proteins and components of the proteasome. These VICE domains appear to function as nuclear protein quality centres during HSV-1 infection. Later in infection, about 5 or 6 hours, ICP27 begins to shuttle between the nucleus and cytoplasm in its role of RNA export protein. ICP27 binds viral RNA through its RGG box RNA binding motif and escorts the RNA to the cellular mRNA export receptor TAP/NXF1[24]. The direct interaction of ICP27, through both the N- and C-termini, with TAP/NXF1 is essential for the export of ICP27 to the cytoplasm and for viral mRNA export.

2.5 Latent infection

Previously described, HSV-1 is a neurotropic virus and, after initial lytic replication in epithelial cells of the primary lesion, the viral progenies enter sensory neurons whose terminal axons innervate the affected area.

HSV establish an infection within sensory nerve ganglia and, once inside the neuron, one or two processes are initiated: either lytic replication (described previously) or repression of lytic genes and establishment of latency. If the lytic genes are not expressed, the viral genome enters a non-replicating nucleosome-associated latent state in the sensory ganglia. The latent infection is characterized by shutdown of virus replicative functions and the inability to detect infectious virus. The virus can remain in this state until a stress stimulus causes some, still unknown, changes that can trigger transcription of the lytic genes.

Sensory neurons are, generally, viewed as the principal reservoir of HSV latency and recent studies suggest that HSV-1 latency may be restricted to define sub-types of sensory neurons [36].

For reasons that are not understood, a true latent state cannot be established *in vitro* and this is one reason because the mouse represents the most commonly used model in which to study HSV latency. The virus will productively replicate in some neurons of the sensory ganglia, while establishing latency in others during the acute phase of the infection, which typically lasts up to 21 days after infection. At 28 days post-infection, infectious virus is generally not detectable and at this point the virus is considered to be latent.

During latency, in the animal models, the lytic viral genes persist in a transcriptionally silenced state, while only the Latency-Associated Transcript (LAT) is transcribed abundantly within approximately one-third of latently-infected neurons. The LAT is a polyadenylated non-coding RNA approximately 8.3-8.5 kb in length. The primary LAT can

be spliced to yield a very stable 2.0-kb intron, and this intron can be further spliced in some neurons to yield a 1.5-kb intron [37].

A number of phenotypes have been associated with the HSV-1 LAT including increasing the efficiency of establishment of latency, reactivation and blocking apoptosis. Studies with LAT^- mutant have suggested that a LAT^- related function may suppress productive-cycle gene expression during acute and latent infection of mouse trigeminal ganglia. LAT mutant containing a partial deletion of LAT, present high neurovirulence and this findings suggest that LAT might protect neurons from being killed by HSV-1, thereby allowing HSV-1 to establish latency in more neurons [37].

While the LAT is the only transcript abundantly transcribed during latency, sensitive RT-PCR analyses have demonstrated that relatively low amounts of some HSV lytic gene transcripts can ben detected during latency [38].

3. Immune response against HSV

Innate and adaptive immunity are both important in determining which HSV-based vector is suitable for a certain strategy of gene therapy [39].

3.1 Immune response to Wild Type HSV

HSV induce innate and adaptive immunity in the infected host. The principal components of innate immunity are macrophages, the complement system, polymorphonuclear leukocytes (PMNLs), natural killer (NK) and innate NK-like T cells (iNKT). Dendritic cells have an essential role and function between innate and adaptive immunity. Adaptive response to HSV infection includes the cellular response mediated by $CD4^+$ and $CD8^+$ T cells and the humoral response by B cells and antibodies [40].

Control of HSV-1 requires recruitment of PMNLs macrophages and NK cells by specific chemokines, and culminates in the induction of antigen specific responses by T cells that control viral replication through lysis of infected cells and cytokine production. Several cytokines have been detected in the infected HSV tissue both in human and in experimental animals. The cytokines detected, during HSV infection, include IFN-α, -β and –γ, IL-1, -2, -4, -5, -6, -10, -12 and -23. IFN-γ production and the presence of $CD4^+$ and $CD8^+$ T cells are essential regulators of viral clearance during acute infection. IL-12 has important roles in the development of T helper cell type 1 (Th1) response and in cell-mediated immunity.

Innate immunity is the first defence against HSV infection by producing and releasing of important cytokines and chemokines following recognition of viral signals via Toll-like receptors (TLRs). TLRs are type 1 transmembrane proteins and they are composed by a variable N-terminal extracellular ectodomain containing 16-28 leucine-rich repeats (LLRs) that are responsible for the detection and interaction with Pathogen-Associated Molecular Pattern (PAMPs) [40]. Different types of glycoproteins are considered PAMPs and are recognized by some TLRs. Only few TLRs were reported to play a role in the recognition of DNA viruses and more particularly, only TLRs-2, -3 and -9 were shown to recognize HSV. TLR2 is expressed on the cell surface and recognizes extracellular ligands. It is able to form heterodimers with either TLR1 or TLR6 and has been shown to play a role in Herpesviruses sensing. HSV-1 interacts with TLR2 and this interaction was described to be detrimental to its host [41].

The role of macrophages in the control of HSV infection consists mainly in phagocytosis of virion and infected cells apoptotic bodies that are recognized by receptors or through the presence of opsonines, such as specific fractions of complement or specific antibodies.

The other mechanisms underlying HSV innate defenses are also apoptosis, and authophagy. Apoptosis is initially triggered and subsequently blocked during a wild-type HSV infection by pro-apoptotic factors including the viral ICP0 gene transcript and others unknown viral or cellular facilitator proteins [42].

Autophagy is a process required for HSV-1 virion degradation and protect against central nervous system (CNS) viral disease and has been shown enhance the presentation of endogenous viral antigens on MHC-I molecules during HSV-1 infection [43].

Dendritic cells have a crucial role and function as a bridge between innate and adaptive immunity by antigen-presenting cells (APCs) function interacting with T cells. The IFN-γ and IL-1 cytokines activate Th1 responses, whereas Th2 responses occur in presence of IL-4 and IL-10. The Th2 pathway will produce a humoral response with a production of antibodies of IgG1 isotype and not inflammatory in nature, in opposite the Th1 pathway typically produce antibodies of IgG2b isotype and activate cytotoxic T cell lymphocytes (CTLs) and a host proinflammatory cytokines that in brain produces lethal encephalitis [44].

3.2 HSV strategies of Immune Response Evasion

Several studies have been shown that HSV encodes different mechanism to interfere with the host immune response, including: nonspecific degradation of host mRNA by RNAse vhs [45], block of DCs activation, inhibition of PKR by U_S11 [46], and γ-34.5 [47]; defective CD1d recycling from the endosome to the cell surface; inhibition of MCH-I peptide loading by ICP47; suppression of interferon response and TLR-dependent inflammatory response by ICP0 [48] and inhibition of apoptosis and interferon. In HSV-1, antiapoptotic activity has been assigned to immediate-early proteins ICP4, ICP27, U_S3, ICP22, gD, and others [49], whereas ICP0 was found to be necessary, and sufficient, to induce the initial apoptosis induction event associated with HSV-1 infection [50]. Granzyme B or Fas caspase-activation and apoptosis have been shown to be inhibited by gJ [51].

3.3 Innate and Adaptive response on Vector Efficacy

Recent studies based on microarray profiles revealed that replicating and non-replicating vectors induce robust antigen presentations.

Innate immune response is critically in the context of oncolytic therapy and appears to be a potent obstacle to achieve tumor destruction through oncolytic vector replication [52].

Innate immune response is followed by the activation of the adaptive response, which is involved in killing infected cells and in production of antibodies directed against viral products. This kind of response also represents a major problem for reduction of effectiveness of HSV-based vector.

Immune responses arising against recombinant HSV vectors can be due to several factors: viral particles components, co-purified packaging cell debris, different routes of delivery, multiple injections of the vectors and low-level *de novo* viral gene product expression.

In several cases, has been observed that pre-existing immune response may reduce the efficiency of the vector but this problem can be eliminate when the HSV-based vector is administered directly into the tumor. Targeted oncolytic viruses must be replicated into the tumor and must destroy the cancer cells, enhancing at the same time, the tumor specific immunity. In particular the CTLs response is very important in oncolytic virotherapy because the viral antigens are presented on the surface of tumor cells. CTLs are subsequently redirected to tumor cell antigens, thereby enhancing the efficacy of oncolytic HSV-1 by inducing antitumor immunity [53]. For these reasons, the inclusion of genes

encoding for various cytokines into viral vectors to enhance antitumor immune responses has been tested and found to be beneficial in several preclinical models of cancer [54].
In several cases, has been observed that pre-existing immune response may reduce the efficiency of the vector but this problem can be eliminate when the HSV-based vector is administered directly into the tumor.

4. HSV-1 based oncolytic vectors

4.1 Different generations of HSV oncolytic vectors

G207 (strain F)	1000bp deletion in both copies of ICP34.5 Disruption of U_L39	None	Recurrent brain cancer (glioma, astrocytoma, glioblastoma) (I, IB and II)	[55]
NV1020 (strain F)	Deletion of one copy of ICP34.5 *tk* under control of viral a4 promoter Deletion of U_L24, U_L55 and U_L56	None	Liver, metastases derived from colonrectal cancer (Phase I)	[56]
OncoVex GM-CSF	Deletion of two copies of ICP34.5 Deletion of ICP27	GM-CSF	Melanoma (Phase III) Head-and-Neck cancer (II/III w Radiotherapy)	[57, 58]
rRp450	Deletion of U_L39	Rat CYP2B1 (cyt. P450)	Liver metastasis, Primary liver cancer (Phase I hepatic arthery	[59]

Table 1: The major Oncolytic HSV-based vectors in human clinical trials.

4.1.1 First-Generation oncolytic HSV-1 Vectors

The first described oncolytic HSV-1 was an attenuated virus mutated in the gene encoding thymidine kinase (*tk*) [60]. Since then, substantial progress has been made in exploiting some of the distinctive features of HSV-1 to engineer efficient and safe oncolytic HSV-1 vectors in the treatment of human cancers. To date, three different HSV-1 vectors, known as G207 [61], 1736 [62], and NV1020 [63], have been moved to the clinic, with encouraging results.

The first generation of vectors contains mutations in single genes that have restricted their replication to dividing cells. Three kinds of these vectors have been constructed: *dls*ptk containing a deletion in the *tk* gene and results in a very neuroattenuated vector; hrR3 containing an insertion of the *E. coli lac*-Z gene in the early gene U_L39, encoding the large subunit of the viral RR (ICP6) [64]; R3616 derived from strain F and HSV1716, derived from strain HSV-1 17+ containing deletions in both copies of the γ–34.5 gene, encoding the neurovirulence factor ICP34.5 [65, 66]. The first type was not pursued in clinical trials because its *tk*-negative status made it resistant to traditional anti-herpetic drugs. The second type has been tested as replicative anticancer vector, alone or in combination with acyclovir/gancyclovir, but hrR3 has shown a high level of toxicity was used at high titers, like a *tk*-mutants. For these reasons they were not considered sufficiently safe to be used in humans. In contrast the deletion of ICP34.5, the neurovirulence factor essential for HSV pathogenicity, provides the greatest attenuation of any individual mutation where the virus can still replicate in actively dividing cells. Although the γ-34.5 deleted vectors were initially conceived for brain tumors due to the neurotropic nature of HSV-1, experimental studies have shown thereafter that they were effective against a wide variety of solid tumors as well as liver metastasis of colon and bladder cancers [67]. NV1020 strain, was the first attenuated HSV virus that relies essentially on the deletion of one copy of 34.5 gene to be constructed and analysed as a viral vaccine in humans and now is being investigated in phase I and II clinical trial for patients with colon cancer that has metastasized to the liver and is resistant to chemotherapy [68]. The results have demonstrated that NV1020 is a safe, novel therapeutic agent for treatment of refractory hepatic malignancy. The use of γ-34.5 mutated viruses demonstrate antitumor efficacy, combined with a good safe profile, and different versions of these vectors are currently in human clinical trials [69, 70] (Tab.1).

However, despite great promise, this first generation of attenuated HSV-1 vectors have serious limitations. The efficacy of the γ-34.5 deleted mutants needs to be enhanced because, like many other attenuated viral vectors, its oncolytic potency seems not to be strong enough to completely eradicate tumors [71].

4.1.2 Second and Third-Generation Vectors

The second-generation vector contains multigenic mutations. These multiple mutation made the reversion to wild type highly unlikely and conferred several important safety advantages. This group contains, G207 presents deletion in both γ-34.5 loci and *lac*-Z gene insertion in the ICP6 gene [72]. Neurotoxicity was further evaluated in non human primates [73]. MGH-1 is an oncolytic HSV-1, which shares the same characteristics of G207 and its oncolytic activity was also evaluated in preclinical models [66].

The G47Δ is a third-generation vector constructed by deletion of U_S12 gene, encoding ICP47 protein, which normally blocks MHC class I-mediated antigen presentation in infected cells. It was tested in human melanoma cells that express higher levels of MHC class I on their surface, and the results have shown a was enhanced stimulation of tumor-infiltrating lymphocytes [74]. The U_S12 deletion also removes the U_S11 promoter, so that U_S11 gene is expressed as an IE gene under the control of U_S12 promoter, thereby suppressing the diminished growth properties of ICP34.5 mutants [74].

Actually, some studies have addressed the possibility of targeting viral replication to specific types of cells or cells that are presenting a peculiar condition. Firstly, the viral gene encoding the essential ICP4 protein was placed under the control of the albumin enhancer/promoter, to target liver cells [75]. However, it is equally clear that further investigations are required to identify both the promoters that will allow the best possible targeting to a particular cancer tissue, and the virus genes whose expression should be targeted in order to obtain those goals. Furthermore, it appears to be possible to target cancer cells at the level of virus entry [76]. This is a very exciting and promising area of HSV-1 research that only very recently has begun to be explored in the context of oncolytic HSV-1 vectors.

4.1.3 Last Generation Vectors: Transgene-Expressing Vectors

To augment the antitumor efficacy of oncolytic HSV vectors they have been modified by the incorporation of expression cassettes for the delivery of various transgenes. These include prodrug-activating vectors, immunostimulatory armed vectors and anti-angiogenic factors. Oncolytic MGH-2 is a vector deleted in U_L39 and ICP34.5 genes and with an insertion of the two-prodrug activating genes, CYP2B1 and secreted human intestinal carboxylesterase [77]. Each of these genes encodes for enzymes that can convert the inactive pro-drugs into their active metabolites. MGH-2 displayed antitumor efficacy against human gliomas cells *in vitro* and *in vivo* when combined with cyclophosphamide and CPT-11, this combination of drugs does not affect oncolytic virus replication. Different studies demonstrate that angiogenesis plays a critical role in tumor development and metastasis. In a study of *Mullen and colleagues* the murine endostatin gene was incorporated into HSV genome. The produced endostatin was found to inhibit angiogenesis in human HT29 colon carcinoma model [78].

Several HSV vectors expressed different interleukins (ILs) and interferons have been constructed. In therapies with different replication-competent vectors expressing ILs has been observed an enhanced tumor-specific killing associated with an increase survival over treatment with HSV alone. Vectors expressing interleukins 4 [79], 12 [80], 10 [81], granulocytes-macrophage colony stimulating factor (GM-CSF) [81], were able to increase tumor immune recognition and reduces the possibility of toxicity derived from the systemic administration of the cytokines. At this regard the use of HSV vectors expressing IL-12 or GM-CSF, two indispensable cytokines for activating dendritic cells (DCs) and boosting the strong immune responses against cancer, seems promising [82]. GM-CSF engenders protective immunity by stimulating the recruitment, maturation, and function of DCs, while IL-12 released from DCs directly primes effector on lymphocytes at local environments, described later. IL-12 also possesses antiangiogenic properties, which may represent a second potential mechanism of its antitumor activity [82]. Several studies have been demonstrated that vectors expressing IL-12 have shown efficacy in both treating the tumor and preventing recurrences after tumor-resection; and use of multiple oncolytic HSV-1 armed with different immunostimulatory genes may be a useful strategy for cancer therapy.

4.2 Interleukin 12

Interleukin-12 (IL-12) is a pro-inflammatory cytokine that induces the production of interferon-γ (INF-γ), favors the differentiation of T helper 1 (Th1) cells and forms a link between innate resistance and adaptive immunity. DCs and phagocytes produce IL-12 in response to pathogens during infection. The activation of the inflammatory/innate response is not pathogen specific, but it is dependent on ligand binding to germline-encoded receptor, for example the Toll-like receptors (TLRs). The family of TLRs is composed of at least ten members, who distributed differentially between inflammatory cells, recognize different classes of pathogen and induce the production of similar patterns of pro-inflammatory mediators.

IL-12 is a heterodimer formed by 35-kDa light chain (p35) and a 40-kDa heavy chain (p40). The light chain has homology to other single-chain cytokines, whereas p40 is homologous to the extracellular domain of members of the hematopoietic cytokine-receptor family. Recently was discovered two other heterodimeric cytokines, IL-23 and IL-27, that are related with IL-12. The IL-12 receptor (IL-12R) is composed of two chains (IL-12Rb1 and IL-12Rb2) that activate the Janus kinase (JAK) – STAT (signal transducer and activator of transcription) pathway of signal transduction. IL-12R is expressed mainly by activated T cells and NK cells. Expression of IL-12R has been shown also on other cell types, such as DCs and B-cell lines. IL-12R is undetectable on most resting T cells, but it is expressed at a low level by NK cells, which probably explains the ability of these cells to respond rapidly to IL-12. Activation of T cells through the TCR (T cell receptor) up-regulates the transcription and expression of both chains of IL-12R, and this up-regulation is enhanced by IL-12 itself, Interferon (IFN)-α, IFN-γ, tumor necrosis factor (TNF) and co-stimulation, through CD28 [83].

Treatment with IL-12 has been shown to have a marked anti-tumor effect on mouse tumors, by inhibiting establishment of tumors or by inducing regression of established of it; the anti-tumors action of IL-12 is complex and uses effector mechanism of both innate resistance and adaptive immunity. Cytotoxic lymphocytes ($CD8^+$ T cells, NK) are often involved in the mechanism of action of IL-12, but their cytotoxic activity is not required in some cases for their anti-tumor activity. IL-12 is know to induce IFN-g secretion by T cells and NK cells, and the possible contribution of IFN-g to the anti-tumor effects of IL-12 have been investigated, because it has been observed that the neutralization of IFN-g, in murine

models, cause a strongly inhibition of anti-tumor effect of IL-12. IFN-γ production, however, is necessary but not sufficient for the anti-tumor effect of IL-12. Recent studies have demonstrated that the IFN-γ and a cascade of other secondary and tertiary pro-inflammatory cytokines, which are induced by IL-12, has a direct toxic effect on the tumor cells and/or might activate potent anti-angiogenic mechanism. Several pre-clinical and clinical models of anti-tumor immunotherapy indicated that IL-12 is a powerful therapeutic agent against cancer. However, systemic expression of recombinant IL-12 protein (rIL-12), currently used in cancer patients; presents disadvantages including cost and dose limitation due to its toxicity.

The ability of the viral approach to deliver a cytokine, locally, combined with IL-12 expression under specific cancer promoter, can reduces the probability of toxicity derived from the systemic administration of these molecules, and can provided important insights for the design of IL-12 combination gene therapy and the improvement of gene vectors for IL-12 therapy [83].

5. Tumor-specific promoters

The fundamental problem for cancer gene therapy is the lack of tumor specificity; proteins that are therapeutic in malignant cells also may be harmful to surrounding normal tissue. One way to circumvent this problem is to use transcriptionally targeted vectors, armed with specific promoters or tissue-specific microRNAs, that can restrict the expression of the therapeutic proteins primarily to cancer cells.

To reduce this toxicity, cancer or tissue-specific promoter systems have been developed by replacing constitutive promoters with promoter/enhancers of cancer/tissue-specific gene markers in the viral vectors [84].

There are two types of promoters, constitutive or inducible. The constitutive promoters can be either of viral origin (cytomegalovirus) or tissue/cancer specific promoters; inducible promoters that have transient expression and can be induced to express trans-genes with hormones, small molecules [85] or can be activated in specific conditions related to tumor microenvironment such as hypoxia. Tumor microenvironment, in human solid tumors, is one of the most important aspects that confer resistant to standard radiotherapy and chemotherapy. Several tissue or cancer-specific cellular promoters have been already used in the context of HSV-1 vectors with encouraging results. From these,

only the albumin enhancer/promoter (AP) shows liver specificity, whereas the E2F-responsive promoter B-myb is activated in most cancers, including liver cancers. Other tumor-specific promoters that have been positively evaluated in oncolytic adenovirus vectors or in HSV-1 vectors, include (a) the HCC-specific α-fetoprotein promoter (AFP) [86], (b) other E2F responsive promoters, including the promoter that drives expression of E2F itself [87], (c) the promoter controlling expression of human telomerase reverse transcriptase (hTERT), which is active in all cancer cells [88] and (d) the hypoxia-responsive promoter (HRE), which is supposed to be active in the hypoxic fraction of solid tumors [89].

To conferring resistance to cancer therapy, recent clinical and experimental studies have suggested that the cellular response to hypoxia can result in dramatic alterations in the expression of a variety of genes, many of which may lead to more aggressive phenotypes. Recent studies have demonstrated that the hypoxia in solid tumors mediates the selection of the cells with diminished apoptotic potential and with genetic alterations, such as loss of p53 tumor suppressor gene or overexpression of the apoptotic inhibitor protein Bcl-2 [90, 91]. Studies of molecular responses to hypoxia have identified the major mediator for cellular hypoxic responses as a transcriptional activator hypoxia-inducible factor 1 (HIF-1). HIF-1 is a heterodimeric basic helix-loop-helix protein consisting of two subunits (HIF-1α and HIF-1β), HIF-1α is the unique O_2-regulated subunit that determines HIF-1 activity. Under hypoxic conditions, HIF-1 binds to the hypoxia-responsive element (HRE) in the enhancer region of its target genes and turns on gene transcription. Several studies have demonstrated the overexpression of HIF-1 in different types of solid tumors [92, 93].

Ruan and colleagues have demonstrated that the number of HRE (3, 6 or 9) increases the level of gene expression; in particular nine copies of HRE elements (9XHRE) increase the gene expression under hypoxic-conditions [90]. An interesting work by *Yeligar and colleagues* has indicated a significant HIF-1α regulation mediated by one peculiar microRNA (miR-199a) in response to ethanol-mediated damage in liver [94].

6. MicroRNA

6.1 Biogenesis of microRNAs

miRNAs are non-coding single-strand (ssRNAs), of 22 nucleotides (nt) in length in their mature forms, that are individually encoded by their own set of genes. Genes for miRNAs are an integral component of the cell genetic program, many of them also being evolutionary conserved.

Actually there are currently 940 identifiable human miRNAs (The miRBase Sequence Database – Release version 15.0).

Studies on miRNA genes distribution throughout the genome have revealed their presence in clusters transcribed as polycistronic primary transcripts, or within regions transcribed as independent units, including intergenic regions, exon sequences of non-coding transcription units or intronic sequences of either protein coding or non-coding transcription units.

Intronic miRNAs orientated in the same direction as the surrounding genes are generally transcribed coincidentally with their host genes and excised by the splicing machinery from the larger transcript in which they are embedded. Indeed, intronic miRNAs may represent a simple way for a protein-coding gene to regulate other protein-coding genes.

The processing of pri-miRNA occurs in the nucleus, through the activity of a double-stranded specific endonuclease RNase II, called Drosha (Fig.6).

Drosha and its partner, the double-stranded RNA binding domain protein DGCR8, cleave the pri-miRNA near a hairpin base and release a 70-90 nt pre-miRNA hairpin-shaped stem-loop precursor.

An alternative pathway for pre-miRNA-like hairpin biogenesis has recently been identified in flies, nematodes and mammalians; this pathway generates new regulatory RNAs from intronic pre-miRNA precursors called "mirtrons", using the splicing machinery and the lariat-de-branching enzyme to bypass Drosha cleavage in initial maturation. Identification of several well-conserved mirtrons in different species indicates their relatively ancient incorporation into cellular regulatory pathways and highlights their likely contribution to animal evolution.

The subsequent export of the pre-miRNA into the cytoplasm is mediated by the exportin-5, a Ran-GTP-dependent nuclear transport receptor. Exportin-5 interacts with the pre-miRNA minihelix motif, which also stabilizes the pre-miRNA structure.

6.2 Processing into mature form, activation and target recognition

In the cytoplasm, the pre-miRNA is processed by the cytoplasmatic ds-RNase III Dicer into an 22 nt miRNA:miRNA duplex with 2 nt overhanging its 3' end. The RNA strand with relatively unstable base pairs at the 5' end is recruited as a single-stranded molecule into RNA-induced silencing complex (RISC). Some of the known proteins that form RISC include: i) Dicer; ii) argonaute (Ago) protein, which has four isoforms of which only Ago2, also knows as "slicer", has the capacity to cleave the target mRNA; iii) PW182 a P-body protein; iv) human immunodeficiency virus transactivating response RNA-binding protein (TRBP), which recruits Ago2 to the complex; and v) fragile X mental retardation protein (FMRP1), which is known to associate with polyribosomes [95]. When assembled into the RISC complex, the mature miRNA sequence targets its complementary mRNA and negatively regulates gene expression by causing either mRNA translation repression or degradation (Fig.6).

miRNAs recognize their target mRNAs mainly through a limited base-pairing interaction between the 5' and region (2-8 nt from 5' end) and the complementary sequences present in the 3'-UTRs of phylogenetically conserved target mRNAs.

The majority of animal miRNAs imprecisely match their targets, thereby causing target mRNA destabilization through "non-slicer" mechanisms, including de-adenylation and other forms of translational repressor.

When targeted for silencing by miRNAs, mRNAs can be concentrated, sequestered from translational machinery, degraded or stored for subsequent use in large macroscopic cytoplasmic foci, named processing bodies (P-bodies). The P-bodies contain a wide range of enzymes involved in RNA turnover, including de-capping enzymes, de-adenylases and exonucleases (Fig.6).

The imprecise matching between animal miRNAs and their targets implies that any given miRNA can bind different mRNAs.

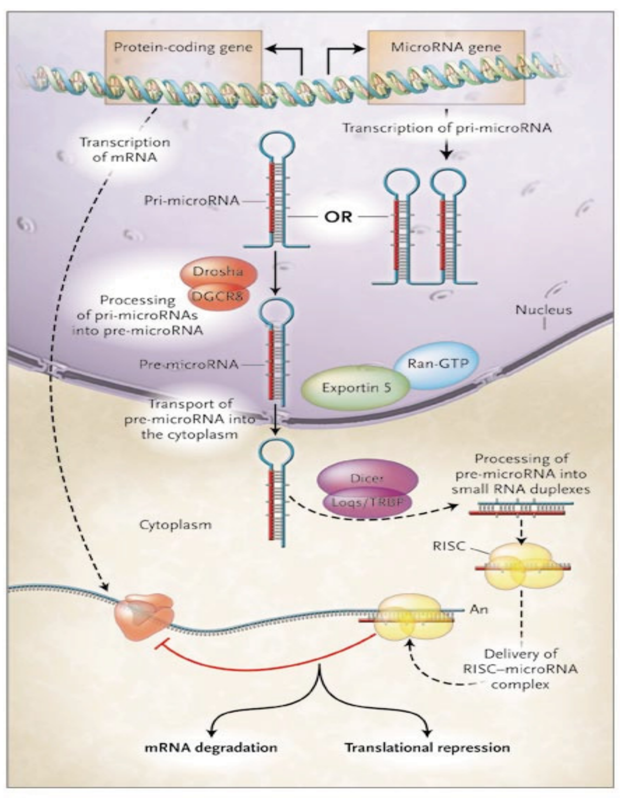


Figure 6: Biogenesis and Regulation of microRNA [95].

6.3 Regulation of microRNA expression

During development, miRNA expression is tissue specific, which means that miRNA may play a role in establishing and maintaining cell type and tissue identity. Different expression profiling analyses have shown that developmental or tissue-specific signalling controls most miRNAs.

Computational approaches have yielded information on transcription and sequential processing of miRNAs in regulatory elements present in the upstream region of miRNA genes. These motifs are preferentially located within 1 kb upstream of the genomic region of protein-coding genes and are evolutionarily conserved. The regulatory binding sites for transcriptional factors are locate upstream of the pre-miRNA gene regions. In addition, their distribution heavily overlaps the predicted transcription-starting site (TSS); miRNA genes may posses more than one predicted TSS. Indeed, a significant fraction of human intergenic miRNAs are characterized by two separate, prominent peaks containing the predicted TSS, with one in the vicinity of the pre-miRNA sequences and the other at the distance about 10 kb. Putative promoters for several human miRNA genes within 0.5 kb

upstream of the pre-miRNA sequences (core promoter regions) are believed to contain essential components for the regulation of gene transcription.
Epigenetic mechanisms, such as DNA methylation, post-translational modification of the histone code and nucleasomal remodelling, contribute to the modulation of gene expression and to the determination of cell and tissue specificity. DNA methylation, a normal process used by mammalian cells to maintain normal expression patterns, is archived by the addition of methylgroups to cytosines within CpG dinucleosides (CpGs), frequently gathered in clusters (CpG islands), by DNA methyltransferase (DNMTs).
Recent evidence suggests that miRNA expression is regulated by epigenetic mechanisms. CpG islands are present both upstream and downstream of genomic miRNAs coding sequences. A significant portion of these CpG islands (40%) also overlaps predicted TSS sites.
A potential oncogenic role of altered miRNA activities has been reported as a consequence of DNA hypermethylation and histone modification of miRNA genomic region [96].

6.4 MicroRNA in Oncolytic Virotherapy

Previously described, HSV oncolytic vectors have been development where different cancer-specific promoter were incorporated in front of an essential viral gene to achieve viral replication and specific killing of cancer cells. However, nonspecific toxicity to normal tissue due to leaky promoters has remained a problem [97]. One approach to achieve this is to take advantage of unique tissue-associated patterns of expression of microRNAs.
As widely know in literature, differential miRNA expression profile are present between normal and cancer cells. Different studies have been shown that some miRNA are overexpressed and some are down-regulated in several cancer cells compared with their normal tissue of origin, suggesting that these miRNA may play a role as oncogenes or tumor suppressor in the tumorigenesis of various human cancer [98].
There are possibly two different strategies that can be used to target cancer by miRNA. The first strategy to target cancer by miRNA is to modify the expression of few deregulated miRNA in tumors using non-lytic viral vectors for miRNA replacement or inhibition. Most therapeutically useful miRNAs seem to be expressed at low levels in tumors but highly expressed, and well tolerated, in normal tissue.
The second strategy is to take advantage of the differential miRNA expression between cancer and non-neoplastic tissue. *Lee and co workers* have demonstrated that inclusion of

specific miRNA target sequence into the 3'-UTR of an essential HSV-1 gene is a viable strategy for restricting viral replication and oncolysis to cancer cells while sparing normal tissue [99]. With this strategy it is possible to increase the tumor specificity and regulate viral replication by increasing the number of copies of miRNA target sequences. In addition, target sequences of more than one miRNA species can be incorporated into 3'-UTR because miRNA expression patterns differ across normal tissue of different origins. Through synergistic effects of various regulatory elements in the promoter 5'-UTR or 3'-UTR stringent regulation of viral gene expression and viral replication will be possible to develop a highly effective and tumor-specific virotherapy for cancer treatment.

7. Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the sixth most common type of malignant cancer in adults, with approximately 630,000 new cases per year in the world. Patients with HCC usually present with tumors in advanced and incurable stages, in fact HCC is the third leading cause of cancer deaths. In the worldwide the bigger risk factors for HCC are chronic infections caused by hepatitis B (HBV) and C viruses (HCV), which increase the risk of developing liver cancer by about 20-fold [100]. These chronic infections, as well as chronic alcoholism, result in cirrhotic conditions that, in more than 80% of cases, evolve in HCC.

7.1 Pathogenesis

Previously described, all etiological factors associated with HCC are caused of chronic liver disease and in particular cirrhotic conditions. In most cases, these cirrhotic conditions are caused by alcoholism and infection by HBV and HCV. In addition, some autoimmune diseases and metabolic disorders, such as hepatic steatosis, may increase the risk of developing HCC [99, 100].

Chronic inflammation is one of the fundamental factors that drive the transition from cirrhotic tissue in pro-carcinogen tissue in combination with external stimuli that induce genetic alterations in mature hepatocytes. The genetic alterations that accumulate in cirrhotic hepatocytes contribute to hepatocarcinogenesis. The HCC cells are mainly characterized by a loss of heterozygosity that includes multiple chromosomes and

mutations in important genes, including *p73, p53, Rb, APC, DLC-1, p16, PTEN, IGF-2, BRCA2, SOCS-1, Smad2* and *Smad4, β-catenin, c-myc* and *cyclin D1* [99].
Angiogenic factors such as vascular endothelian growth factor (VEGF), the platelet-derived growth factor receptor (PDGFR), and fibroblast growth factor (FGF) are released by the tumor, moreover inflammatory cells and tumor stromal cells participate in HCC neovascularization. Like other malignancies, HCC is characterized by an imbalance in growth promoting and regulation signals, and the mitogen-activated protein kinase (MAPK) cascade. The process of tumor angiogenesis increase the number of receptors required for MAPK cascade signalling, thereby helping to regulate the proliferation, differentiation and survival of tumor cells.

7.2 Treatment of HCC

Before the better understanding of the anatomy of the liver, potentially curative therapies for HCC was reduced by partial hepatectomy and liver transplantation, the latter only possible in patients with early stage tumors, which accounts for less than 15% of patients.
The local ablative therapy (example microwave coagulation therapy) and transarterial techniques (example interstitial radiotherapy) are not effective for HCC treatment because of their high toxicity in cirrhotic patients.
In the last two decades, there has been an increase in the use of resection as the treatment modality of HCC, thanks to the diagnostic advances that have allowed earlier and earlier diagnosis of this disease. But even in these cases, local recurrence and *de novo* tumour appearance in other areas of the cirrhotic liver compromises the long-term survival.
Recently developed molecularly targeted drugs improve survival of about three months over placebo and are not free from side effects.
In this regard oncolytic virotherapy offers a promising therapeutic option for treating HCC and in particular oncolytic vectors based on HSV-1 offer an exciting new modality in the armamentarium of anti-cancer agents. These vectors have the potential to infect and selectively replicate within cancer cells-sparing normal cells. The specificity of oncolytic viruses in HCC therapy depends upon interplay of the intrinsic properties of these viruses and cellular alterations of transformed cells [101].

7.3 miRNA involved in HCC

HCC tumor development is thought to develop in a multi-step process requiring the accumulation of several genetics and structural alterations and affecting many different pathways [102]. It has been suggested that many of miRNA changes that occur during hepatocarcinogenesis do so early, so that many changes that predispose to HCC have already taken place in liver cirrhosis and other pre-malignant lesions [103].

Subsequent changes the in miRNA expression in the transition from cirrhosis to HCC seem to be less marked [103].

A miRNA expression profiles indicates a several pattern of microRNAs that are upregulated (oncomiRs) and downregulated (tumor suppressor) in HCC. The overexpression of miR-221 and miR-222 results in up-regulation of the tumor suppressor and cell cycle regulator p27 (Kip1), recently shown, a new family of oncogenes targeting p27 (Kip1) [104]. miR-100 has been found to be altered in breast, lung and ovarian cancers [105]. Additional miR-224 [106], miR-21 [107] and let-7a [108, 109] are overexpressed in HCC, the overexpression of miR-17-92 cluster (which comprises seven miRNAs) results from aberrant transcription and activation of c-Myc. It is known that miRNAs from miR-17-92 cluster act as oncogens by influencing the translation of E2F1 messanger RNA [110]. miR-10b is another miRNA upregulated in HCC, it promotes cell migration and invasion [111].

In opposite, down-regulationof miR-126 is strikingly associated with HCC, by excessive alcohol consumption [112]. This miRNA is a tumor suppressor not only in HCC but also in breast cancer. In both malignancies also the lower expression of miR-145 leads the normal parenchyma to mammary carcinoma [113] and HCC, respectively. Predicted target sites for miR-145 are MAPK transduction proteins such as MAPK, MAP4K4, MYCN, FOS YES and FLI-I [114].

Another down-regulated miRNAs are miR-198, miR-199a and miR-200a, which are also under-expressed in in colonic adenocarcinoma [115, 116].

The miR-199 and miR-200 families have are also related to liver fibrosis and the expression of miR-199a* was silenced in several proliferating cells excluding fibroblasts [115]. In according of this the treatment of cancer cells with miR-199a or miR-199a* (derived from the same precursor) was associated with promotion of apoptosis. The mechanism underlying apoptosis induced by miR-199a was caspase-dipendent, whereas a caspase-indipendent pathway was involved in apoptosis induced by miR-199a expression. *Seonhoe and colleagues* have identified that the MET proto-oncogene as a target of miR-

199a* and, very interestingly, extracellular signal regulated kinase 2 (ERK2) was also down-regulated by miR-199a*. Studies indicates when introduced into tumor cells, miR-199a* induced pronounced apoptosis, suggesting that miR-199a* is a putative tumor-suppressor [117]. From these studies is clear that the expression of miR-199a* was almost completely silenced in different examined tumor cell lines derived from lung, skin, breast, prostate, leukemia, pancreas in comparison with normal cells. A pancreatic adenocarcinoma cell line, called BxPC3, presents an aberrant expression of c-MET and ERK2, both associated with cell proliferation, growth and migration [118]. From the microRNA database miR-199a resulted down-regulated in this cell line.

Down regulation of miR-199a, miR-199a* and miR-200 in liver injury tissue was associated with the hepatocarcinogenesis [115]. miR-199a* is also one of the negative regulators of the HCV replication [119].

Deregulation of selected miRNAs is associated with an alterated response of tumor to commonly used chemotherapeutic agents. For example, miR-214 induces cell survival and cisplatin resistance through targeting the PTEN/Akt pathway, while inhibition of miR-21 and miR-200a increase sensitivity of the cells to gemcitabine [120, 121].

Purpose

Purpose

Oncolytic virotherapy is an emerging experimental treatment platform for cancer therapy. HSV-1 vectors have many advantages over other oncolytic viruses owed to several unique features of the HSV-1 life cycle. First, HSV-1 is a well-studied large DNA virus with a genome of 153 kbp, encoding at least 89 proteins, with unique genetic flexibility. Many viral proteins are non-essential for the multiplication of the virus in cultured cells, therefore permitting to replace the virus genome with foreign DNA, yet still leave the virus to replicate in the appropriate cellular environment. Furthermore, multiple genes associated with virulence can be deleted or modified without affecting the virus capacity to replicate, within and destroy tumor cells during its lytic phase. Second, HSV-1 possesses powerful inherent cytolytic potential for most types of cells, including murine cells, thus allowing evaluating both the toxicity and safety of HSV-1 vectors in murine cancer models. Third, anti-herpetic drugs (acyclovir, forscanet) are available and provide a safety mechanism in case undesired local or systemic infection occurs. Finally, HSV does not integrate into the cellular genome, remains in an episomal state, so it cannot cause insertional mutagenesis.

The overall objective of this thesis was to develop safe and efficient HSV-1-derived oncolytic vectors, designed to strictly target and eradicates human hepatocellular carcinomas (HCC), the most common liver cancer of adults.

A first modification was ***targeting viral replication,*** in the way to direct viral replication, to hepatocellular carcinoma by introducing, into HSV genome, a target sequence for a specific microRNA that is known to be down-regulated in these tumor cells. The miRNAs carry out their function by regulating the transcription of the gene located upstream respect to its target sequence.

Different studies are suggesting that cancer types can be clustered based on microRNA expression; some microRNA resulted up-regulated in a peculiar type of cancer, but down-regulated in others. We focused our attention on microRNA199 (miR-199) that was resulted abundantly de-regulated in a HCC cell and associated with epatic-tissue transformation after liver transplantation [128], to generate a target-replication HSV-1 oncolytic vector. We have engineered the HSV-1 genome by targeting expression of the immediate early viral protein 27 (ICP27), an essential gene absolutely required for HSV-1

replication. Four copies of microRNA-199 target sequence were inserted at the 3'-UTR between the stop codon and poly(A) region, of U_L54 gene, codifying for ICP27.

The second part of the work was focused on ***vector enhancement***. We have armed this miR-regulated HSV-vector with the coding sequence for Interleukin-12 (IL-12) a cytokine abundantly known for its anti-tumor activities. The expression of the IL-12 was regulated by human cytomegalovirus promoter (HCMV) to generate a positive control and by a hypoxia-responsive element promoter (9XHRE) to allow its expression only in the tumor cells.

The generated vectors were evaluated *in vitro* to demonstrate the ICP27 regulation through miR-199 target sequences and the IL-12 expression based on tumor promoter specificity, while *in vivo* were evaluated the biodistribution, the toxicity and the anti-tumor effects in mice models.

Materials and Methods

Materials and Methods

Plasmids used and constructions

The EGFP, luciferase and mIL12 cytokine transgenes were derived from commercial plasmids pIRES-EGFP (Clontech), pGL3Luc (Promega) and pOFRmIL-12 (InVivogen) respectively. The HSV-1 sequences that were used in this project were obtained from a set of cosmids that have been previously described [122] and were cloned into pBluescriptSK (Stratagene) or pTZ18U (InVitrogen) plasmids (see Results section).

All the DNA fragments were excised with New England Biolabs (NEB) enzymes and purified from agarose gel with Millipore Kit (LSKGEL050) after electrophoresis run.

All the fragments obtained by PCR were done by amplify the regions of interest with DNA polymerase High-Fidelity (Phusion Hot Start, FINNZYMES) (see Results section).

Commercial plasmids	Brand
pIRES-EGFP	Clontech
pGL3 Basic (Luc) vector	Promega®
pBSSK plasmid	Stratagene®
pTZ18U plasmid	InVitrogen®
pOFRmIL-12	Invivogen®

Table 1. Commercial plasmids used for the subsequent cloning.

pgl3LucmiR199 plasmid (constructed and kindly given by Massimo Negrini's group)

Four tandem copies of perfectly complementary sequences to miR-199 in the 3'UTR of the *firefly* luciferase expression cassette were cloned into the pGL3 vector (Promega) at the XbaI restriction site.

The oligonucleotides containing the sequences: 1(5'CTAGATAACCAATGTGCAGACTACTGTccTAACCAATGTGCAGACTACTGTccTAACCAATGTGCAGACTACTGTccTAACCAATGTGCAGACTACTGTccT-3') and 2(5'CTAGAggACAGTAGTCTGCACATTGGTTAggACAGTAGTCTGCACATTGGTTAggACAGTAGTCTGCACATTGGTTAggACAGTAGTCTGCACATTGGTTAT-3'), were preaviously annealed and then phosphorilated using a PNK kinase (Roche).

Bacterial transformation and plasmid purification

The plasmids obtained after ligations were used to transform MAX efficiency® DH10B™ competent cells (InVitrogen®), using the chemical transformation procedure suggested by the manufacturer. Each transformation was spread on separate broth (LB) agar plates containing a specific antibiotic (depends on plasmid resistant) and plates were incubated at 37°C overnight.

Single colonies from these plates were picked and inoculated as started cultures in 5 ml LB medium containing the appropriate selective antibiotic. After an overnight incubation at 37°C, mini-preparative of plasmid were processed with Plasmid Mini kit (QIAGEN®). The extracted DNAs were checked and the positive colonies were amplified and diluted 1/100 into LB selective medium to produce a plasmid maxi preparative with a Plasmid Maxi kit (QIAGEN®).

Recombinant vector construction by BAC "recombineering" method in bacteria cells

The cloning of large DNA virus genome, such as that of HSV-1 as bacterial artificial chromosome (BAC), has facilitated the easy construction of recombinant viruses by homologous recombination in *Escherichia coli* [123].

A HSV-1 (strain F) genome cloned into a BAC, flanked by loxP sites, has been maintained in SW102 bacteria, a D*galK* specific strain [123, 124]. The BAC-HSV genome was modified by a *galK*-based selection in these specific bacteria. As shown in Fig. 1, primers of 20 bp were designed of 5' and 3' *gal*K expression cassette plus 50 bp of HSV homology sequence, suited for the locus where the deletion or the insertion is desired. Subsequently the *gal*K expression cassette, flanked by the 50 bp of homologous HSV sequences, was amplified by PCR.

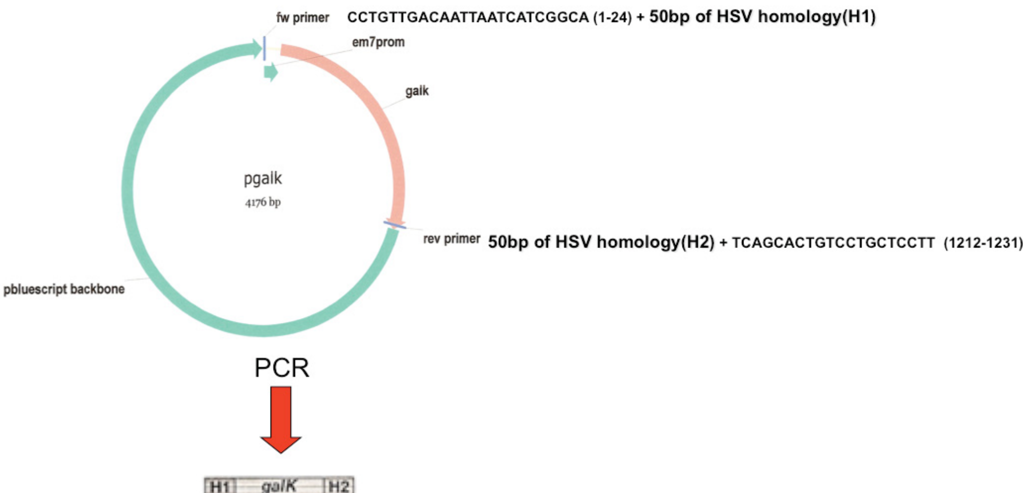


Figure 1. pgalK plasmid with primers and HSV (H1 and H2) homology sequences, the fragment indicated was amplified by PCR reaction.

The obtained PCR fragment was inserted in HSV-genome by electroporation technique in SW102 bacteria [124] (Fig. 2) and the *gal*K positive colonies were evaluated by screening on MacConkey-galactose added plates: only the bacteria that have acquired the fragment were able to growth on the medium.

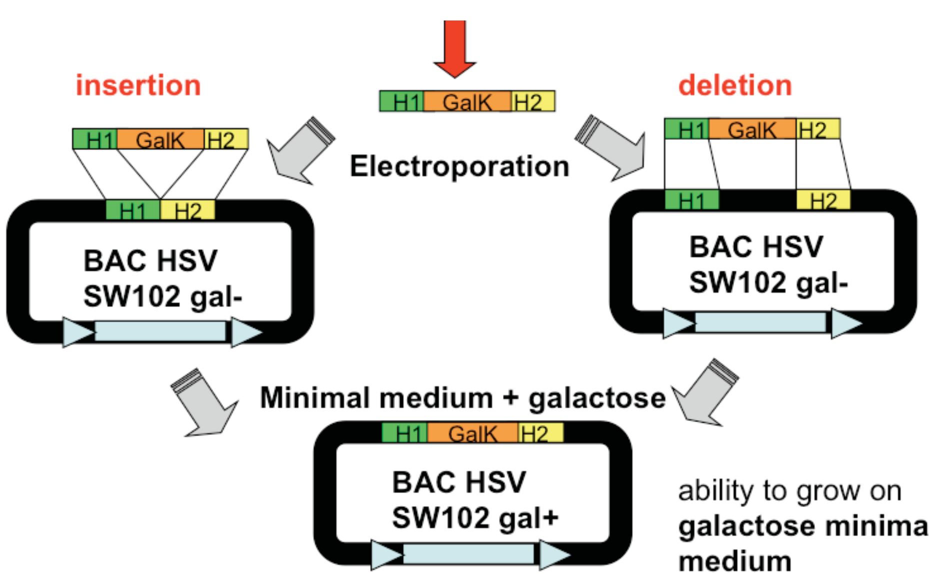


Figure 2. In the panel the insertion of *gal*K cassette into BAC-HSV genome by bacterial electroporation.

In the second step the *gal*K gene was substituted with a specific interest gene expression cassette with the same 50 bp of HSV homology sequences used for the *gal*K insertion. As done previously, the expression cassette of interest gene was amplified by PCR reaction and subsequently was inserted by electroporation in bacteria. To evaluate the substitution

between the *gal*K gene and the interest gene, the *gal*K negative colonies were grow on 2-deoxy-galactose medium added with glycerol (DOG). The SW102 bacteria were growing in LB medium, the DNA was extracted and purified with a QIAgen Miniprep kit (QIAGEN®) and screened with appropriate restriction enzymes digestion (Fig. 3).

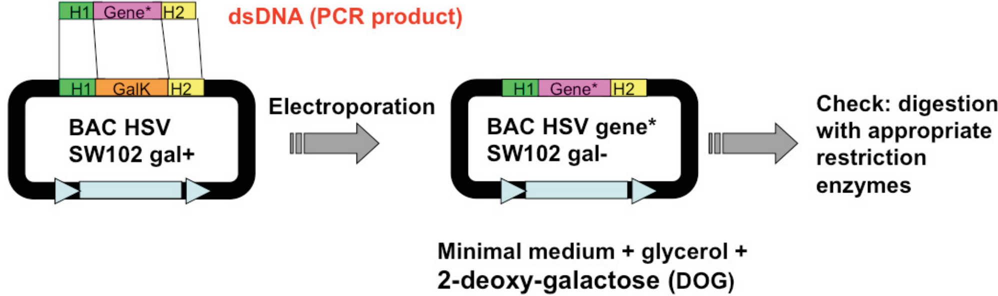


Figure 3. Recombineering technique for the excision of *gal*K expression cassette and insertion of a specific gene expression cassette**.**

In this last step the BAC was deleted from the HSV genome, by Co-transfection with HSV-BAC DNA and a Cre-Recombinase expression plasmid on Vero cells, as shown in figure 4. To evaluate the excision of the BAC, the samples were analyzed by Southern blot and specific PCR reaction, while the presence of the interest gene was evaluated by Southern blot, Western blot and biological activity, where possible.

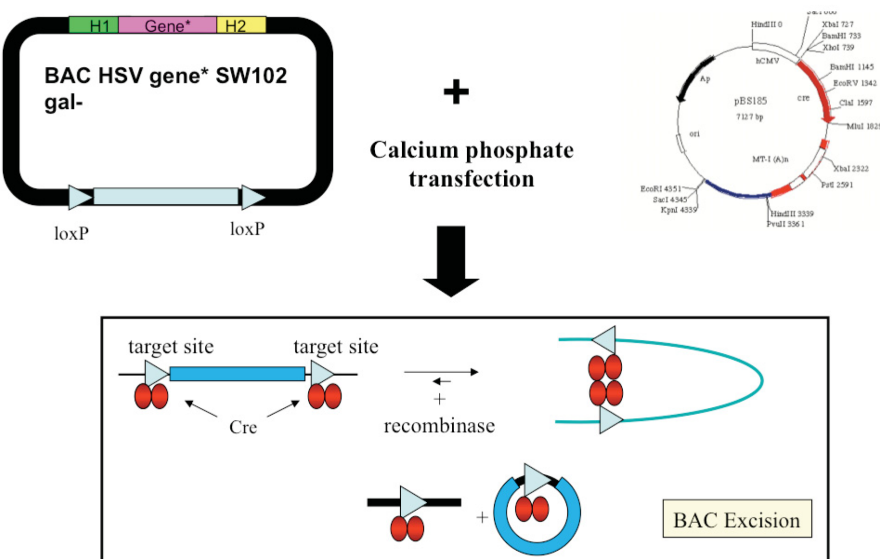


Figure 4. Co-transfection with HSV-BAC DNA and Cre-Recombinase expression plasmid to eliminate the BAC from the HSV genome.

Cell lines and culture conditions

Vero (ATCC, Rockville, MD) and *Vero 2.2* cells, (that are stably transfected with HSV-1 ICP27) were maintained in high glucose Dulbecco's modified Eagle's medium D-MEM (EuroClone) supplemented with 2mM L-Glutamine, 100 units/ml Penicillin, 100 mg/ml Streptomycin and 10% Fetal Bovine Serum (USA approved, EuroClone). Vero 2.2 cells were monthly subjected to selection with 1 mg/ml of Geneticin (G418, Roche).

Vero-derived *7b* cells (expressing the HSV-1 immediate early gene products ICP4 and ICP27 required for virus replication), were fifty-days subjected to selection with 700mg/ml of Geneticin (G418, Serva).

HepG2 human hepatoma cells (ATCC, Rockville, MD) were maintained in MEM (EuroClone) supplemented with 1X Non-Essential Amino Acids (NEAA, EuroClone), 2mM L-Glutamine, 100 units/ml Penicillin, 100 mg/ml Streptomycin and 10% Fetal Bovine Serum.

HepG2miR-199 cells have been obtained by stably transfecting HepG2 cells with a miR-199 expressing plasmid (kindly given by Massimo Negrini). These cells have been constantly subjected to selection with 700 mg/ml of Geneticin (G418, Roche).

BNL 1NG A.2 murine cells derived from BNL CL.2 (ATCC, Rockville, MD) transformed with nitrosoguanidine, were maintained in high glucose D-MEM supplemented with 2mM L-Glutamine, 100 units/ml Penicillin, 100 mg/ml Streptomycin and 10% Fetal Bovine Serum (GIBCO).

BxPC-3 human pancreatic adenocarcinoma cells (ATCC, Rockville, MD) were maintained in RPMI 1640-Medium (GIBCO) supplemented with 2mM L-Glutamine, 100 units/ml Penicillin, 100 mg/ml Streptomycin and 10% Fetal Bovine Serum (GIBCO).

Recombinant vector construction by homologous recombination in eukaryotic cells

Alterations of the HSV-1 genome in eukaryotic cells can be achieved in a number of ways. These usually require a process in which portions of the virus genome, which have been cloned into plasmid vectors, are first modified *in vitro*; then the modified sequences are introduced into the virus genome through homologous recombination in cultured cells [125] and recombinant viruses are selected.

Calcium-phosphate method

The recombinant vectors HSVLucgJHE, HSVLuc27*tmiR199*gJHE and HSVLuc27*tmiR199* HCMV or 9XHRE mIL-12 were generated by homologous recombination with co-transfection by calcium - phosphate method. A solution of 500 ml of HBS (HEPES 200mM, NaCl 135 mM, KCl 5mM, dextrose 5mM and Na_2HPO_4 0,7 mM) at pH 7,05 were added with 5 mg of viral DNA co-transfected with 1 mg of linearized plasmid DNA and 30 ml of 2M $CaCl_2$. The calcium-phosphate-DNA suspension was transfer drop by drop into 60 mm culture plates (Corning™) with a 70% confluent cell monolayer. After ten minutes of incubation at 37°C, was added D-MEM with 2% FSB (EuroClone) and the transfected cells were incubated at 37°C in 5% CO_2 for five-six hours. Finally the cells were washed three-times with D-MEM (EuroClone), were subjected to treatment with glycerol for three minutes and were washed again for four-times to eliminate the toxic residues of glycerol. A 3ml/well of complete D-MEM with 2% FSB (EuroClone) were added to the cells and they were incubated at 37°C in 5% CO_2 until the appearance of viral foci, generally 3-4 days later.

Limiting dilution

This method is useful to isolate and purify the recombinant viral vectors with the advantage to avoid the contamination of possible recombinants with parental virus. The stock of virus obtained after calcium-phosphate co-transfection was used to isolate the recombinant viruses. A suspension of $2x10^6$ cells in a final volume of 2 ml D-MEM without FSB was infected with 20-30 pfu of the virus stock and incubated, in a rotation movement, for 1 hours at 37°C, to permit the virus adsorption. Subsequently, 8 ml of D-MEM with 10% FSB was added to the cells and they were plated in a 96 well flat-bottom plates (NuncClone®), in a volume of 100 ml/well and incubated at 37°C until the appearance of plaque. A wells that containing a single plaque were identified, marked and the plate was freeze at -80°C and thaw at 37°C in incubator. All identified-single plaque were scraped and collected into a 1,5 ml tube (Eppendorf) and freeze at -80°C. After the cells were plated in a 24-48 well plates and infected with each single virus to extract the viral DNA, which was characterized by Southern blot.

All viral vectors were derived by three limiting dilution cycles and Southern blot characterization to evaluate the presence or absence of the specific insert.

Viral DNA extraction and Southern blot

Viral DNA of different HSV-modified vectors have been isolated from several plaques and characterized by restriction enzyme and Southern blot analysis, as described below. The cells were infected with HSV-vector at MOI of 1; 24 hours later they have been collected and centrifuged for 15 minutes at 13000xg. The supernatant was removed and the pellet was re-suspended in 200 ml of lysis buffer (10mM Tris-HCl pH8, 10mM EDTA) containing 0,25 mg/ml of Proteinase K (EuroClone) and was placed in agitation at 37°C for five hours or overnight. Afterwards DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1 Ambion, Applied Biosystem) and chloroform-isoamyl alcohol (24:1 Ambion, Applied Biosystem), centrifuged each time for 3 minutes at 9000xg. A double volume of cold absolute ethanol was added to the aqueous phase containing DNA, and precipitated at -80°C for 30 minutes. The DNA was centrifuged at 13000xg for 15 minutes and the supernatant was removed. The obtained pellet was washed with 200 ml of cold 70% ethanol and centrifuged at 13000xg for 3 minutes, air dried and re-suspended in sterile water (the water volume depends of the pellet). The resultant viral DNA of mutants were digested with appropriate restriction enzymes and the viral DNA bands could be seen within the cellular smear on 1% agarose gel electrophoresis. The DNA was transferred in a Hybond-N+ nylon membrane (Sigma-Aldrich) and the size of regions of interest was determined by hybridization with an appropriate probes conjugated with peroxidase (RPN 3005 kit, Amersham Life Science) and were revealed with ECL detection reagent (Thermo Scientific).

Viral stock production and titration

Once isolated the desired recombinant virus, it is useful to produce a viral stock, in order to have large quantity of recombinant virus with high concentration of plaque forming units (pfu).

HSV-1 midi stocks were prepared by infecting $4x10^7$ Vero 2.2 cells to produce HSVD27gJHE and Vero cells to produce HSVLucgJHE, HSVLuc27*tmiR199*gJHE and HSVLuc27*tmiR199*HCMVmIL-12 and 9XHREmIL-12, at a multiplicity of infection (MOI) of 0.01. Infected cells were harvested when a 100% cytopathic effect was reached. Infected cells were separated from supernatant, subjected to three cycles of freeze-thawing, sonicated and centrifuged at 1700xg. The resulting supernatant from the treated pellet was added to the one obtained in the previous step and centrifuged at 48000xg to recover the

virus. The virus was further purified by density gradient centrifugation (OptiPrep, Life Technologies, Inc.) and re-suspended in cold-sterile PSB-A 1X (EuroClone) [126]. Virus stocks were stored at -80°C. HSV-1 recombinants titres (in plaque forming units – pfu) were determined by plaque formation assay.

Plaque Assay

Different cell lines were infected with the HSV-modified vectors. At specific time points (8, 12, 24, 48 and 72 hours) the cells were scraped, collected with supernatant, centrifuged at 400xg for 10 minutes and sonicated for 30 seconds. A confluent monolayer of Vero cells was infected with serial dilution of each sample (generally 10^{-2} to 10^{-10}) and incubated for 1 hour at 37°C in constant low agitation. After this, the medium was discharged and were added 3 ml/well of 3% methylcellulose (Sigma-Aldrich) (3g powder methylcellulose were dissolved in 300 ml of medium with 2% of FBS), and incubated for 2-3 days in incubator at 37°C in 5% CO_2.

Subsequently the plates were fixed in 2 ml/well of 1% crystal violet solution (50:50 methanol: dH_2O v/v) for 10-20 minutes. Were counted the number of plaques per well and determined the average for each dilution (each dilution was done in triplicate), the obtained number was multiply by factor of 10 to get the number of plaque forming units/ml (pfu/ml) for each dilution.

In vitro Luciferase Assay

$4x10^4$ cells were infected with the recombinant HSVLuc viruses and seeded in a 96-well plates (NuncClone). 24 hours post infection; the cells were incubated with Cell Culture Lysis Reagent (CCLR, Promega) for 10 minutes at RT. The lysate was placed in a 96-well dark plate (NuncClone). After reading the blank, 100ml/well of Luciferase Assay System (E4550, Promega) were added to the samples. The plates were analyzed by VICTOR^3 Multilabel Plate Counter (PerkinElmer, USA) at 562 nm.

Western blotting

$5x10^4$ HepG2 and HepG2miR199 cells were infected with the recombinant vectors at MOI 0,1 or 0,01. Following 1 hour adsorption, the infected cells were plated in 48 well plates and incubated for 8, 12, 24 and 48 hours at 37°C in 5% CO_2. The samples were collected and submitted to electrophoresis on 10% SDS-polyacrylamide gels. Proteins were

transferred to polyvinylidene difluoride membranes (BioRad) and incubated with a mouse monoclonal antibody specific for HSV-1 ICP27 protein (Virusys) diluited 1:1000 and with a rabbit antibody specific for actin (Sigma) diluted 1:3000, followed by a goat anti-mouse and goat anti-rabbit IgG horseradish peroxidase-linked secondary antibody at 1:4000 dilution, respectively. Immunocomplexes were detected by ECL detection kit (Thermo Scientific).
Band signals were acquired using densitometry Quantity One software (BioRad) and the ratio between the ICP27 and the corresponding b actin bands was used to quantify ICP27 modulation by miR-199.

Cell proliferation assay with Tetrazolium salt

HepG2 and HepG2miR199 cells were infected in suspension for 1 hour at MOI 0,01 – 0,1 or 1 and dispensed into 96-well flat-bottom plates at a concentration of $2x10^4$ cells/well. Cells were then incubated at 37°C in 5% CO_2; cell viability was evaluated at different time points (1 to 6 days) with a XTT assay kit (Roche). Absorbance values were read at 450 nm by a microtiter plate spectrophotometer (Multiscan EX 1.1 Labsystem).

Cell viability assay with Crystal-violet

$5x10^3$ cells were dispensed in 96-well plates flat-bottom (NunClon™) in final volume of 100 ml/well in 10% FBS medium. 24 hours after, the medium was discarded and the cells were infected with the different recombinant vectors and 200 ml of 2% FSB medium was added. After five days the medium was discarded, the cells were washed with D-PBS and fixed with 50 ml/well of 0,5% Glutaraldehyde for 10 minutes and washed again twice-times with D-PBS. In each well was added 50 ml of Crystal-violet and fixed for 20 minutes, washed with D-PBS and air-dried the plates overnight. 100 ml of 10% Acetic-Acid were added in each well and the plates were analyzed with ELISA reader (Thermo Scientific) at 595 nm to performed the viability of the cells.

Enzyme-Linked Immunosorbent Assay (ELISA) for mIL-12

The infected cells were scarpered, collected in a 1,5 ml tube and centrifuge at 1200xg for 5 minutes and the supernatant was placed into a clear tube. The mIL-12 expression was evaluated by ELISA kit IL-12 mouse (p70) BD Pharmigen®.

Coating with anti-mouse IL-12 (quantity indicates by manufacturer) was generated in coating buffer (Na_2HPO_4 and NaH_2PO_4) and placed in 96-well plates for 1 hour at 37°C. The plate was washed three-times with 300 ml/well of PBS + 0,05 % Tween 20 (Serva) and added 200 ml/well of blocking buffer for 1 hour at RT. The plate was washed again with the same solution.

The standards were prepared in a series of 8 two-fold dilution in blocking buffer, with 4000 pg of mIL-12/ml to obtain the calibration patron line and the samples were diluted in the same blocking buffer solution. Three-washed was made and a volume of 100 ml/well of each samples was placed in a 96-well plate and incubated at RT for 2 hours. Five-washed was made again and were added 100 ml of working solution (avidine HRP-coniugated) and incubated for 1 hour at RT. Washed again for seven-times and added 100 ml/well of substrate solution (ABTS) and incubated for 20 minutes in the dark. Color reaction can be stopped by adding 50 ml/well of H_2SO_4 2N solution and the plate was red with ELISA Reader (Thermo Scientific) at 450 nm.

Mice and tumor models

Balb/c and *athymic* (*nu/nu*) female mice (5-8 weeks old) were purchased from Charles River Laboratories (Barcelona, Spain). For subcutaneous tumor formation a total of $2x10^6$ BxPC-3 cells, were injected in the left hind flank. Tumor size was monitored at indicated time points by measuring two perpendicular tumor diameters using a precision caliper. Tumor volume was calculated using the following formula:

$$V = (\text{length x width})^2 \text{ x } 0{,}5$$

Survival was checked daily and mice were euthanized in their general status deteriorated or subcutaneous tumors exceeded 20 mm in diameter.

Recombinant vectors and treatment procedure

Balb/c and *nu/nu athymic* mice were administered intravenously by tail vein of $2x10^6$ infection units (IU) of HSVLucgJHE and HSVLuc27*tmiR199*gJHE mixed in a total volume of 100 ml saline solution. *Athymic nu/nu* mice were administered by intra-tumor injection of different doses of HSV-recombinant vectors mixed in a total volume of 50 ml saline solution (see Results section for the doses).

Bioluminescence imaging

Anesthetized animals received the substrate D-luciferin (Promega®) by intraperitoneal injection of 150 mg/kg. Ten minutes later, light acquisition was performed in an IVIS CCD camera system (Xenogen, Alameda, CA, USA) and analyzed with Living Image 2.20 software package (Xenogen). Typically, a circular region on interest measuring 3 cm in diameter was defined in abdomen of mice, and quantification of light emission was performed in photons/second.

Statistical analysis

All experiments data are expressed as mean and standard deviation.

Statistical significance was assessed using the two-way ANOVA and Bonferroni post-test for cytotoxicity $P<0,05$ was considered statistically significant.

Results

Results

TARGETING VIRAL REPLICATION

In the first part of the thesis we have addressed the possibility of targeting viral replication to specific types of tumors.

Plasmids construction

In order to develop recombinant vectors that have been used in this work, we have constructed different plasmid with Herpes sequences in order to recombine them into the viral genomes. All the plasmids that have been constructed in this project have been checked by DNA digestion with specific enzymes and controlled by electrophoresis gels.

Construction of pBICP27, pBICP27-XbaI and pBICP27tmiR-199 plasmids

The 2421 base pair (bp) of BamHI-SacI fragment from HSV-1 genome (nucleotides 113323 to 115743) containing U_L54, corresponding to entire ICP27 sequence, which is an essential HSV-1 immediate-early protein (IE), was cloned into the BamHI-SacI sites of pBSSK plasmid (Fig.1 panel A). Two PCR fragments were generated from pBICP27 as DNA template in order to insert the XbaI restriction site in the 3'-UTR (position nucleotide 115275) between the ICP27 stop codon and the poly (A) sequence (Fig.1 panel B). Subsequently the PCR products containing the XbaI restriction site were used to replace the StuI-SacI sequence from pBICP27 achieving pBICP27-XbaI plasmid. XbaI restriction site was confirmed by restriction enzyme digestion and the ICP27 restored sequence was confirmed by sequencing analysis (data not shown).

The 100 bp XbaI fragment containing four copies of miR-199 target sequence, excised from pGL3miR199, was ligated within pBICP27-XbaI site to obtain the insertion of the miR-199 binding sites into the ICP27 3'-UTR (pBICP27-miR199). The presence of the inserted sequence was confirmed by sequencing analysis (data not shown).

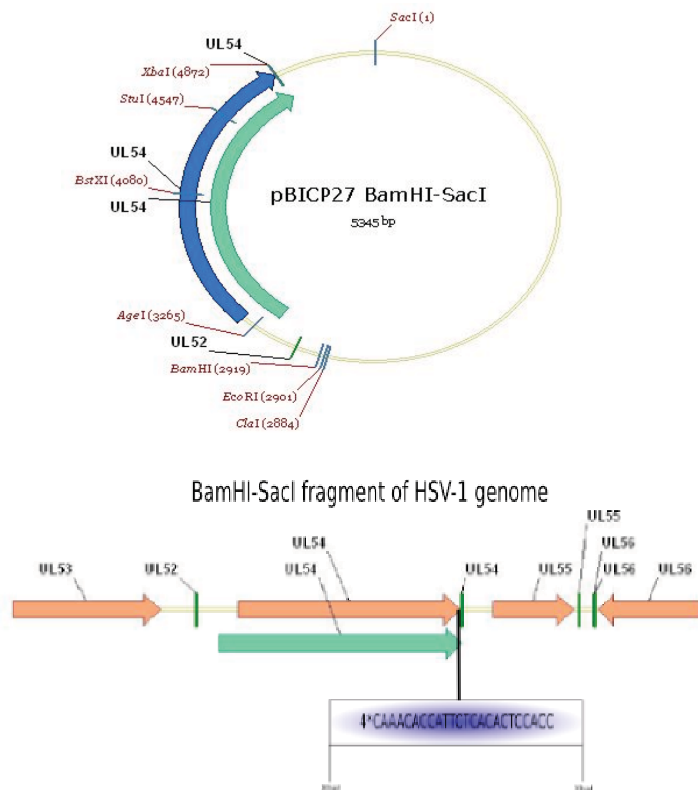


Figure 1. Panel A) Fragment of HSV-1 was cloned the BamHI-SacI site of pBSSK plasmid, to generate plasmid pBICP27 BamHI-SacI. Panel B) the fragment derived from HSV-1 genome with indicates the target sequences for miR-199 flanked by XbaI sites.

Construction of pgJ1 and pgJHE plasmids

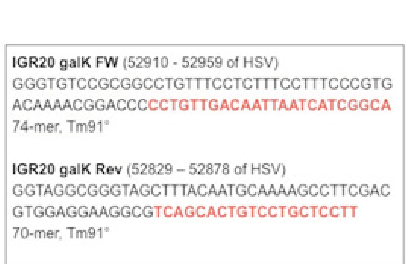
The 2036 bp of SalI-HindIII fragment from the HSV-1 genome (nucleotides 136308 to 138345) containing U_S5 *locus*, which encodes for a non-essential glycoprotein J, was cloned into pTZ18U plasmid. The resulting plasmid, pTZgJSalI-HindIII was used to generate pTZgJHE, containing a deletion in U_S5 between the TATA box and the gJ coding sequence (nucleotides 137626-137729), by insertion of the EGFP coding sequence driven by the cytomegalovirus (CMV) promoter into NruI-SphI sites.

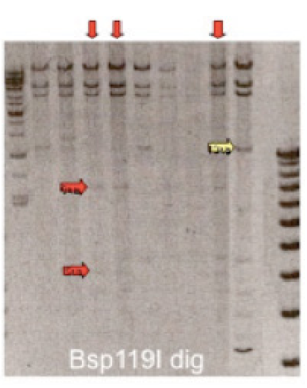
Construction of recombinant HSV-1 vectors

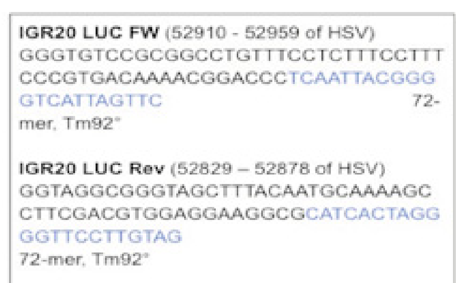
Construction of HSVLuc

The HSV-1 vectors, named HSVLuc, has been generated from a pre-existing HSV-BAC (F-BAC) containing a full-length infectious clone of HSV-1 with the BAC (bacterial artificial chromosome) vector inserted into the intergenic region between U_L3 and U_L4 [123]. HSVLuc recombinant vector (Fig.2 panel A and panel B) was done in bacteria, using the "recombineering" system previously described in Material and Methods section. By this way the PCR fragments, expressing *gal*K as first step (Fig.2 panel A) and substituted, as second step, by pHCMV-Luciferase-BGHpolyA cassette (Fig.2 panel B), were introduced by homologous recombination in *E.coli* using the *gal*K positive and negative selection procedure into the non-coding HSV-1 intergenic region 20 (IGR20), between U_L26.5 and U_L27 HSV-1 sequences (HSV-1 map position 52878-52910) (Fig.2 panel C). HSVLuc possess the ICP27 gene sequence and expresses the wild-type immediate early (IE) protein.

All the viruses that were used in the thesis are based on this backbone recombinant virus that is expressing luciferase in the IGR20 intergenic region (between U_L26 and U_L27) for the *in vivo* evaluation of toxicity, virus biodistribution and oncolytic effect.







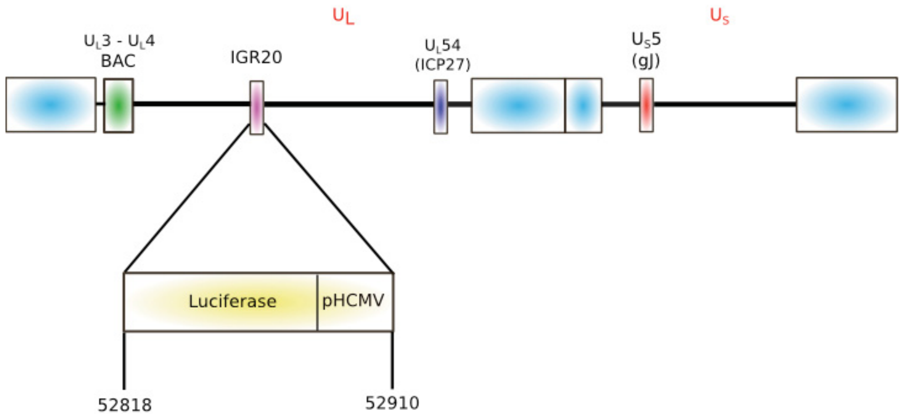


Figure 2. Panel A) Designed IGR20 *gal*K primers and electrophoresis gel that indicate the bacterial positive colonies, checked by Bsp119I enzyme digestion (yellow 5 kb and red 7 kb arrows). Panel B) Designed IGR20 *gal*K primers and electrophoresis gel that indicate the bacterial positive colonies, checked by Bsp119I enzyme digestion (yellow and red arrows). Panel C) Schematic representation of BAC-HSVLuc genome.

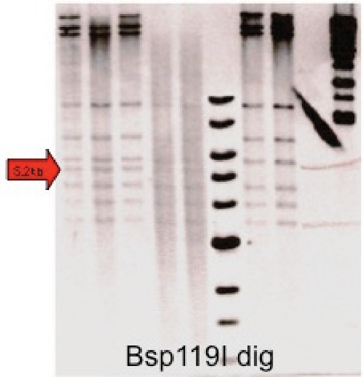
HSV-1LucΔ27 vectors

HSVLucΔ27 vector (Fig.3), was constructed through the completely deletion of ICP27 coding sequence starting from the BAC-HSVLuc genome. ICP27 is an immediate-early protein essential for viral replication that regulates the synthesis of early (E) and late (L) proteins. Deletion of this gene leads to an abortive infection with no yield of progeny virus.

To generate recombinant defective vector deleted in ICP27 gene, we have designed primers of 20 bp of 5' and 3' *gal*K expression cassette + 50 bp HSV ICP27 homology sequences (Fig.3 panel A and B). SW102 bacteria cells were transformed by electroporation with BAC-HSVLuc vector and *gal*K PCR fragments as described in Material and Methods section. The ability of these bacteria to grow on galactose minimal medium was restored when *galK* expression cassette was recombined into a BAC-HSVLuc vector.

ICP27 galK FW (113.451- 113.497 of HSV)
ATA CAT GCC ACG TAC TTA TGG TGT CTG
ATT GGT CCT TGT CTG TGC CGG AG**C CTG**
TTG ACA ATT AAT CAT CGG CA

ICP27 galK Rev (115.227-115.278 of HSV)
TTG TAC CTA AAA CAG GGA GTT GCA ATA
AAA ATA TTT GCC GTG CAC GTA CGG G**TC**
AGC ACT GTC CTG CTC CTT



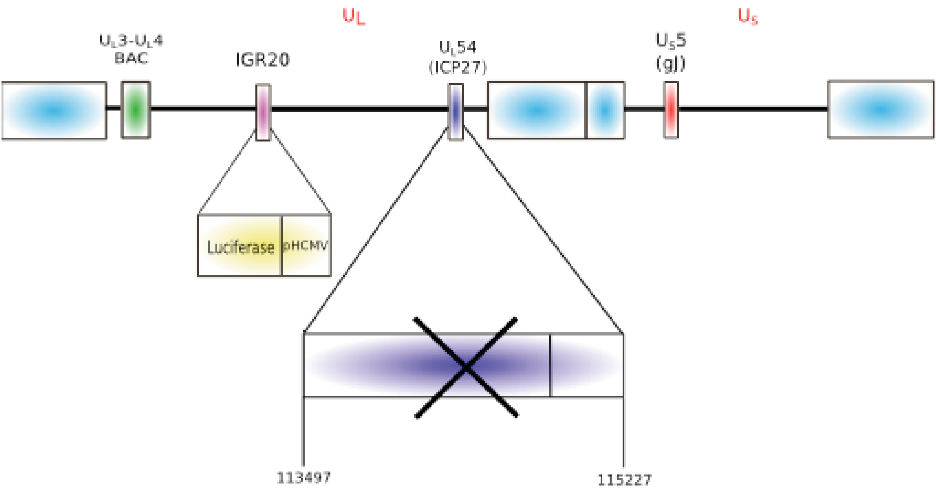


Figure 3. Panel A) Designed U_L54 *gal*K primers and electrophoresis gel that indicate the bacterial positive colonies, checked by Bsp119I enzyme digestion (red arrow). Panel B) Schematic representation of BAC-HSVLucD27 vector generated by "recombineering" system.

HSVLucgJHE and HSVLucΔ27gJHE recombinant vectors

The insertion of the green fluorescent reporter gene has been introduced in all the based vectors to follow the infection *in vitro*, by fluorescence microscope. HSVLucgJHE and HSVLucΔ27gJHE recombinant vectors containing HCMV-EGFP cassette into the HSV-1 U_S5 region were constructed starting from HSVLuc and HSVLucΔ27 DNA viral vectors, previously devoided of BAC sequence, as described in Material and Method section. The recombinant viral DNAs and the pgJHE plasmid, previously mentioned, have been co-transfected respectively in Vero cells for HSVLuc or in which are complementing cells for ICP27 deletion, for HSVLucΔ27 vector deleted on ICP27, using the standard calcium phosphate transfection method ($CaPO_4$). The recombinant viruses (HSVLucgJHE and HSVLucΔ27gJHE) were identified by isolation for GFP fluorescent plaque phenotype under the fluorescent microscope. Recombinant viruses were purified through three rounds of limiting dilution and the presence of the transgene, in the appropriate locus, was verified by Southern blot analysis. The protein expression from the recombinants was evaluated by immunofluorescence.

HSVLucgJHE vector represents the positive control while HSVLucΔ27gJHE vector represents the negative control vector for all the experiments.

HSVLuc27tmiR199gJHE recombinant vector

In the last years, several studies have revealed that the expression of miRNAs is deregulated in human HCC in comparison with matched non-neoplastic tissue. Members of the miR-199 family emerged in several studies as frequently down regulated in HCC, ovarian, lung and pancreatic cancer. Based on these observations and based on microarray analysis in patient with primary and recurrent HCC where miR199a expression have been shown to be down-regulate [128], we have developed a novel strategy for targeting HCC by taking advantage of the differential miR-199 expression level between normal and cancer cells.

In this study we have constructed a miR199-controlled HSV1-based oncolytic vector to test the ability of miR199 to regulate ICP27 gene expression. Rescuing the modified ICP27 gene, which contains four copies of the miR-199 target sequence into 3'-UTR, was generated the replication target HSVLuc*27tmiR199*gJHE vector (Fig.4). The ICP*27tmiR199* binding site expression cassette was introduced in U_L54 locus of the HSVLucΔ27gJHE genome by homologous recombination by co-transfecting HSVLucΔ27gJHE DNA and pBICP*27tmiR199* recombinant plasmid, previously described, in Vero cells. Because of the essential role of ICP27 protein in HSV-1 replication, only recombinant vectors expressing ICP27 were able to grow in Vero cells. The presences of ICP27 gene and miR-199 binding sites in HSVLuc*27tmiR199*gJHE recombinant viral genomes were confirmed by Southern blot and by sequencing analysis (data not shown).

The recombinant virus was purified through three rounds of limiting dilution and the ICP27 expression, on different cell lines with specific monoclonal antibodies, was verified by Western blot techniques, afterward described.

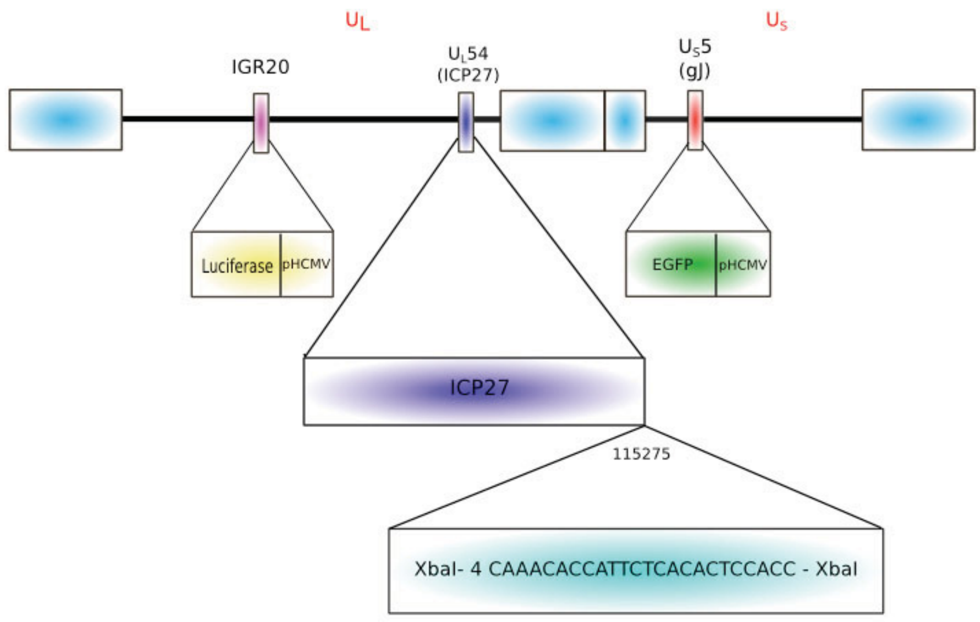


Figure 4. Schematic representation of HSVLuc*27tmiR199*gJHE.

HSVLuc27tmiR199gJHE recombinant viral replication is inhibits in cell expressing miR199

We have compared the replication capacity of HSVLuc27*tmiR199* in a panel of human HCC-derived cancer cell lines (HepG2, Hep3B, Huh-7), HepG2 engineered cells constitutively expressing miR-199 (HepG2miR199, Material and Methods) and normal cells (HUVEC, primary fibroblasts and primary hepatocytes). Here we have shown that, in infected HepG2 and HepG2miR199 cells, the viral replication can be regulated by miR-199a expression. HepG2 and HepG2miR199 cell lines were infected with HSVLucgJHE (positive control), HSVΔ27gJHE (negative control) and HSVLuc*27tmiR199*gJHE vector at MOI 0,1 and 0,01. Effect of miR-199 in the ICP27 target expression and subsequently on viral replication was monitored at 24 and 48 hours post-infection directly by fluorescence microscope (Table 1 a and b) and measured by viral yield in plaque formation assay (Fig. 5).

MOI 0,1 24 hrs	No Infect	HSVLucD27gJHE	HSVLucgJHE	HSVLuc27 *tmiR199*gJHE
HepG2				
HepG2 *tmiR199*				

MOI 0,01 24 hrs	No Infect	HSVLucD27gJHE	HSVLucgJHE	HSVLuc27 *tmiR199*gJHE
HepG2				
HepG2 *tmiR199*				

Table 1a. Fluorescence microscope photography of HepG2 and HepmiR199 infected by HSV modified vectors at indicated time points

MOI 0,1 48 hrs	No Infect	HSVLucD27gJHE	HSVLugJHE	HSVLuc *27tmiR199*gJHE
HepG2				
HepG2 *tmiR*199				

MOI 0,01 48hrs	No Infect	HSVLucD27gJHE	HSVLugJHE	HSVLuc 27*tmiR199*gJHE
HepG2				
HepG2 *tmiR*199				

Table 1b. Fluorescence microscope photography of HepG2 and HepmiR199 infected by HSV modified vectors at indicated time points.

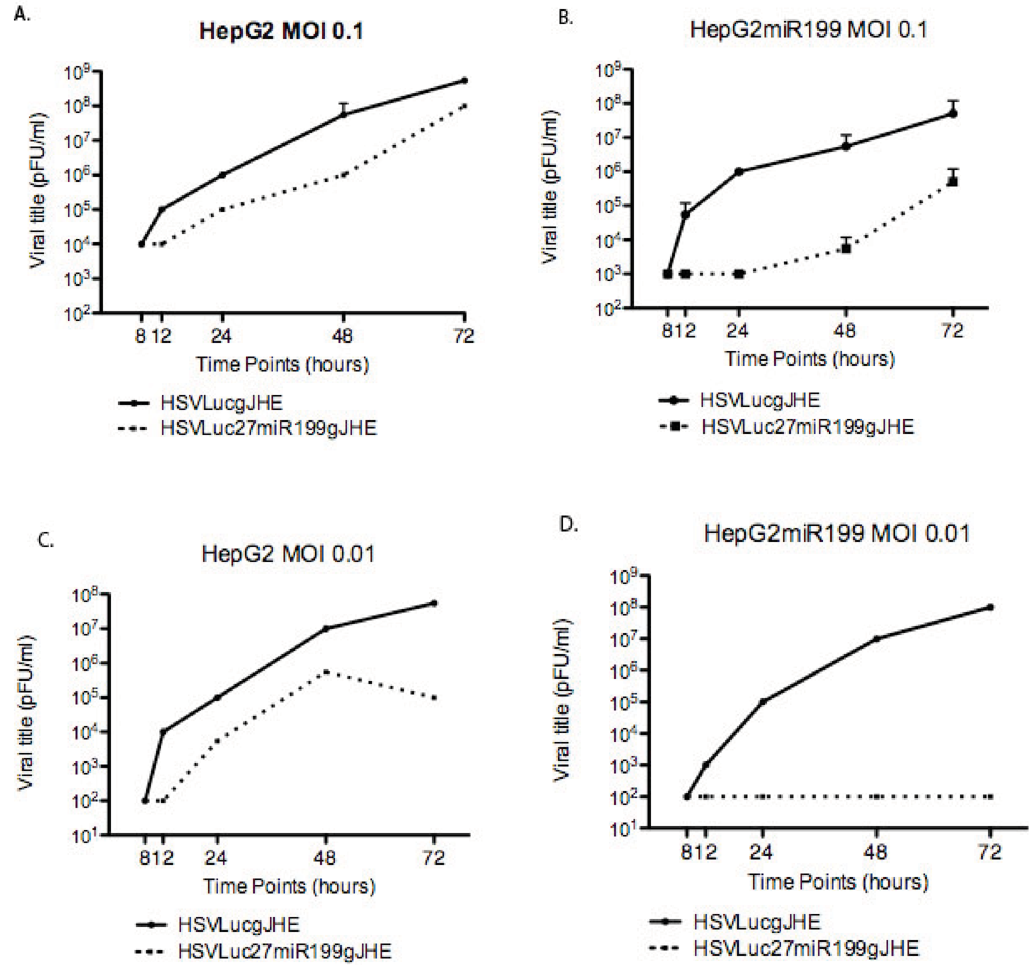


Figure 5. Yield of viral progeny on HepG2 (panel A and C) and HepG2miR199 (panel B and D) at different time points post-infection.

As expected, in the HepG2miR199 cell line, expressing miR-199, infected with HSVLuc*27tmiR199*gJHE the viral yield was 5/6-fold less than the same cells infected with the HSVLucgJHE recombinant control virus, as is shown in figure 5 panel B and D; in comparison, no significant differences in virus yield were observed from HepG2 infected with HSVLuc*27tmiR199*gJHE or HSVLucgJHE viruses, figure 5 panel A and C. These results are showing that the expression of miR-199, which is constitutively express in the engineered HepG2miR199 cells, was able to, almost, complete inhibit the ICP27 expression and consequently to drastically reduce viral replication. The differences in viral yield from HepG2miR199 cells infected with 0,1 or 0,01 MOI of HSVLuc*27tmiR199*gJHE over the time were due to the amount of viral particles infection. From these data, confirmed by previous results with cells infected also at 1 MOI (data not shown), we are demonstrating that the regulation of ICP27 expression was dose dependent and the amount of miR-199a produced

by HepG2miR199 cells was not sufficient to completely block all the ICP27 expression in cells infected with 0,1 MOI, leading to a small amount of viral protein that was sufficient to initiate lytic viral replication which was evident after 48 hours post infection, figure 5 panel B.

Expression of ICP27 protein is closely regulated with target miR-199a

Western blot assays were performed in order to investigate and to determine if miR-199a was capable to bind the miR-199 target sequence downstream of ICP27 3'-UTR and if this RNA was able to regulate the expression of the HSV essential viral gene.

HepG2 and HepG2miR199 cells were infected at MOI of 0,1 or 0,01 with HSVLuc*27tmiR199*gJHE recombinant virus, HSVLucgJHE and HSVLucΔ27gJHE as positive and negative control, respectively. We have observed (Fig.6) a strong expression of ICP27 in HepG2 cell lysates infected with both HSVLucgJHE (indicated as F) and HSVLuc*27tmiR199*gJHE (indicated as miR) vectors and in HepG2miR199 infected with HSVLucgJHE (F), whereas the expression of ICP27 protein was totally absent in HepG2miR199 infected cells with HSVLucΔ27gJHE vector (indicated as D27) and in HepG2 or HepG2miR199 not infected cells (indicated as Ni). As expected, the expression of ICP27 gene was suppressed by miR-199 through a direct action on the 3'-UTR target sequences present in the gene.

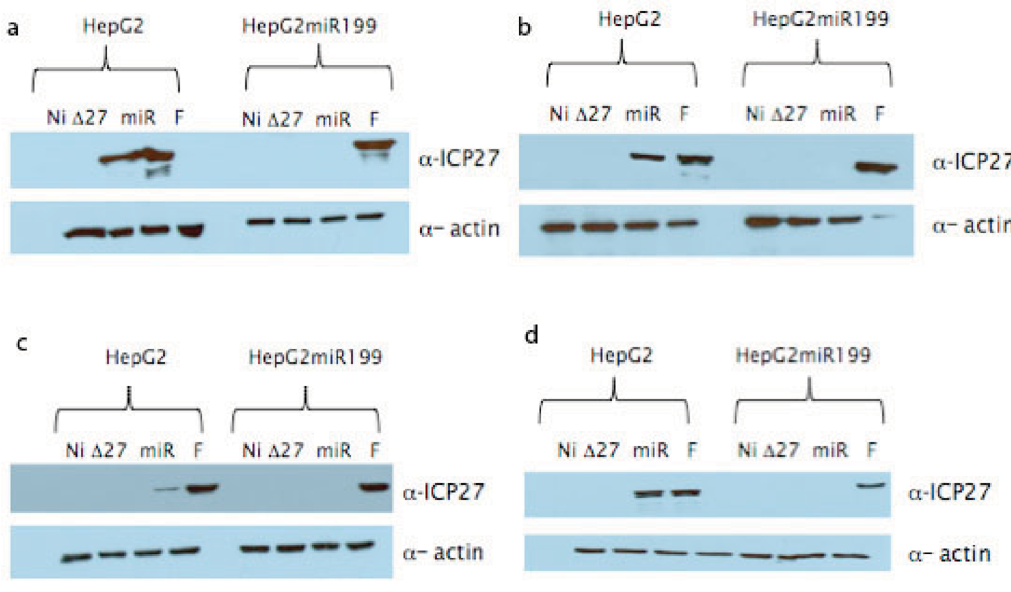


Figure 6. Detection, by Western blot, of ICP27 protein in the cell lysates from HepG2 and HepG2miR199 infected with different HSV-1 modified vectors. Lysates at 24 (left) and 48 (right) hours post-infection, at MOI 0,1 (a, b) and 0,01 (c, d), respectively. Positive samples controls were detected with specific anti-actin monoclonal antibody.

miR-199 prevents cytotoxicity by inhibiting miRNA-regulated HSV virus

To further investigate the effects of miR-199a in the regulation of viral replication and consequently in the cytolytic activity of HSVLuc*27tmiR199*gJHE compared to HSVLucgJHE vector, we have measured the HepG2 and HepG2miR199 cell viabilities after infection by MTT assay. Results have shown a highly significant, time-increasing, cytotoxic effect on HepG2 cells infected with HSVLucgJHE or HSVLuc*27tmiR199*gJHE, as expected (Fig. 7).

No toxicity was seen in HepG2miR199 cells infected with HSVLuc*27tmiR199*gJHE comparable to the negative controls HSVLucΔ27gJHE or not infected cells, whereas a strong cytolytic effect was evident in HepG2miR199 cells infected with the HSVLucgJHE control vector. These data are confirming that the inhibition of viral replication is exclusively due to the presence of miR-199 target sequences downstream the ICP27 gene associated with the presence of miR-199a complex expressed by HepG2miR199 cells.

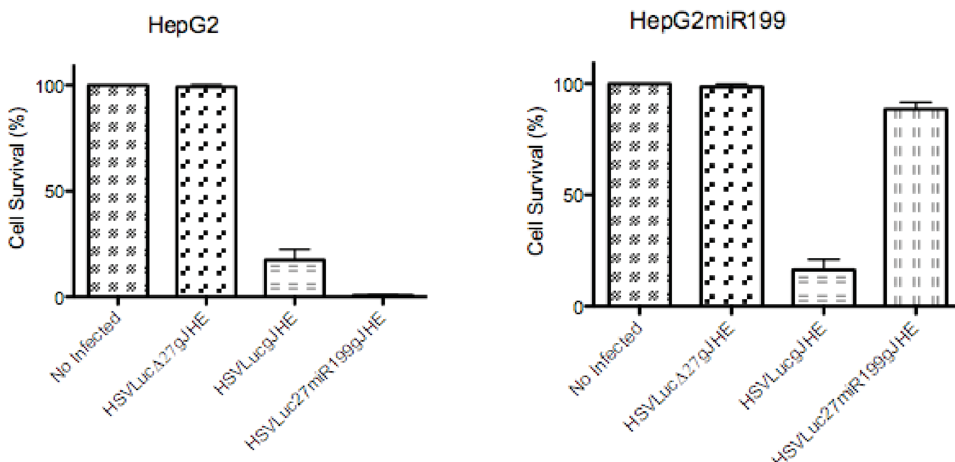


Figure 7. MTT assay for HepG2 and HepG2miR199 no infected cells and infected with HSV-modified vectors at MOI 0,01.

Evaluation of biodistribution and replication in immunocompetent and nude mice

Vectors with or without the target miR-199 sequences were tested *in vivo*, to check the viral biodistribution, replication and toxicity in normal tissue.

The mice were divided in different groups, by virus dose, as indicated in table 2.

Group of mice	Dose of HSVLucgJHE/mouse	Dose of HSVLuc27*tmiR199*gJHE/mouse
Bal.a (4 mice)	1x10^{7}	
Bal.b (4 mice)		1x10^{7}
Bal.c (4 mice)		1x10^{8}
Nud.a (5 mice)	2x10^{6}	
Nud.b (5 mice)		2x10^{6}

Table 2. Balb/c and *nude* mice divided in different groups.

12 Balb/c and 10 *athymic nu/nu* female mice were inoculated intravenously, by tail vein injection, with different doses of HSVLucgJHE and HSVLuc*27tmiR199*gJHE and were monitored by luciferase through *in vivo* IVIS scan bioluminescence detection.

In Balb/c mice both viruses are showing (Fig.8) the same biodistribution at 24 hours post injection that it was detectable in the liver-abdominal area. The replication of HSVLuc*27tmiR199*gJHE was detectable mainly at 24-48 hours post infection with a

maximum emission of photons in 10^7 at 24 hours post-injection (Fig. 8), while, the HSVLucgJHE control vector replication was stronger than HSVLuc*27tmiR199*gJHE and was still detectable at more than 5 days post-infection.

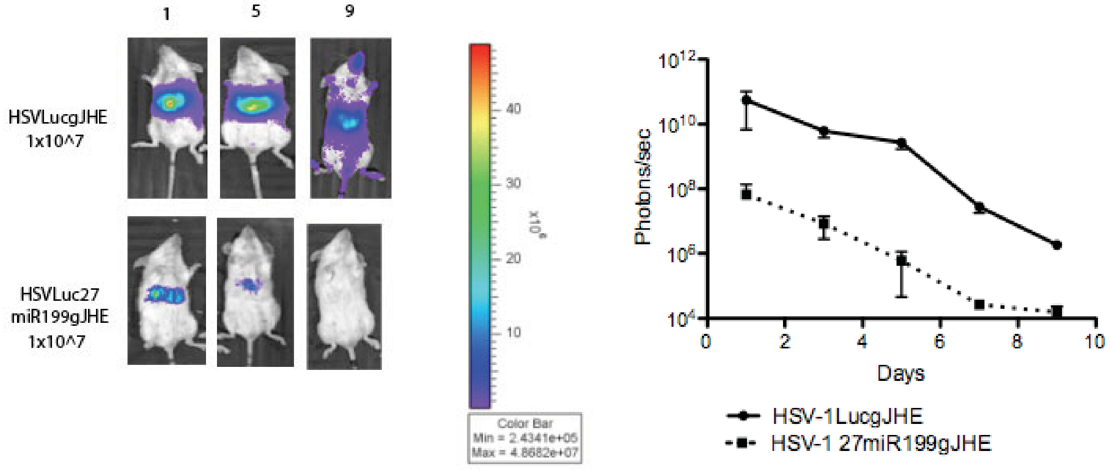


Figure 8. IVIS scan image of Balb/c mice infected with HSV-modified vectors at indicated dose (group Bal.a and Bal.b). The images and graphic shown the luciferase detection based on viral replication at different days in the same mouse.

The injection of 1x10^7 pfu of HSVLucgJHE have demonstrated to be lethal in Balb/c mice, while the animals that have received HSVLuc*27tmiR199*gJHE vector have well tolerated a dose that was 10 times higher than the control virus (data not shown).

The same studies were performed in the *athymic* mice to evaluate the same parameters. 10 fold less amount of recombinant vectors were inoculated intravenously since these animals were more susceptible to virus infection compared to Balb/c mice. The animals were followed for 40 days after viral injection with IVIS scan to evaluate the viral biodistribution, replication and toxicity. The figure 9, on the left side, is showing the photon emission average between HSVLucgJHE and HSVLuc*27tmiR199*gJHE, also confirming in these animals the HSVLucgJHE vector replication. This data was associated with mortality, in fact, as shown in figure 9, right graph, at the same dose, the vector HSVLuc*27tmiR199*gJHE results more attenuated than the HSVLucgJHE.

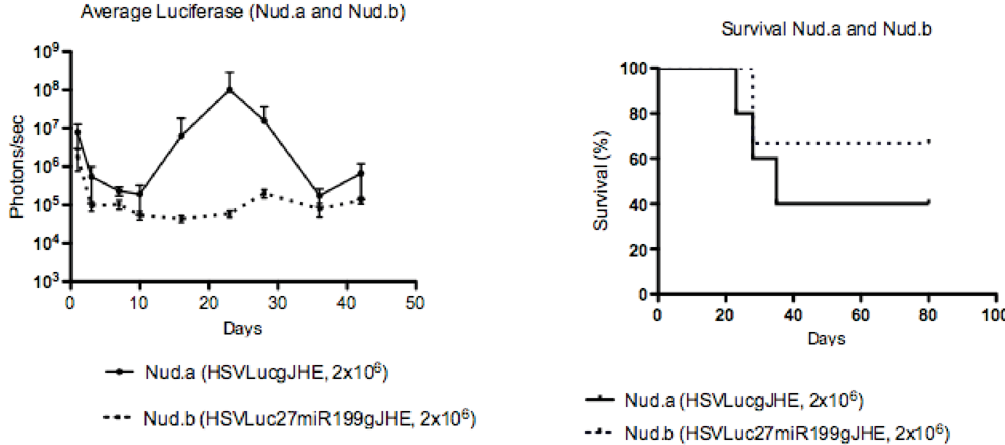


Figure 9. In the left side the graphic indicates the photon/emission in a treated animals, while in the right side graphic indicates the animals mortality.

HepG2 cell lines are not tumorigenic in nude mice and did not used in vivo

In order to evaluate the efficacy of the HSV vector expressing ICP27 miR-199 target sequences compared with the control vector expressing the wild type ICP27, *in vivo*, we have decided to check different tumour cell lines since HepG2 cells were not able to give tumour mass in a murine model [129]. Three different cell lines (Huh-7, Hep3B and BxPC-3) derived from human hepatocarcinoma and pancreatic adenocarcinoma and two cell lines (BNL and CT-26) derived from murine hepatoma and colon-carcinoma, were analysed to evaluate the HSV-infection susceptibility, replication and cytotoxicity.

$1x10^6$ of each cell line was infected with HSVLucgJHE (positive control) and HSVLuc*27tmiR199*gJHE at MOI 0,1; the susceptibility of these cells to HSV infection was evaluated by GFP expression after 24 hours post-infection with fluorescence microscope (Table 3). These data indicate that Hep3B and CT-26 were very low susceptible to the HSVLucgJHE and HSVLuc*27tmiR199*gJHE vectors infection while the Huh-7, BxPC-3 and BNL cells have shown to respond positively to HSV infections.

Based on these results we have decided to exclude Hep3B and CT-26 from our studies. We have evaluated the HSV vectors viral replications only in Huh-7, BxPC-3 and BNL infected cells by plaque-forming units assay, and the cytolytic effect by cell viability using Crystal-violet assay (see Material and Methods).

Virus	Hep3B (Human)	Huh-7 (Human)	BxPC-3 (Human)
HSVLucgJHE			
HSVLuc 27*tmiR199*gJHE			

Virus	BNL (Mouse)	CT-26 (Mouse)
HVSLucgHJE		
HSVLuc 27*tmiR199*gJHE		

Table 3. Different cell lines susceptibility to HSV-modified vectors.

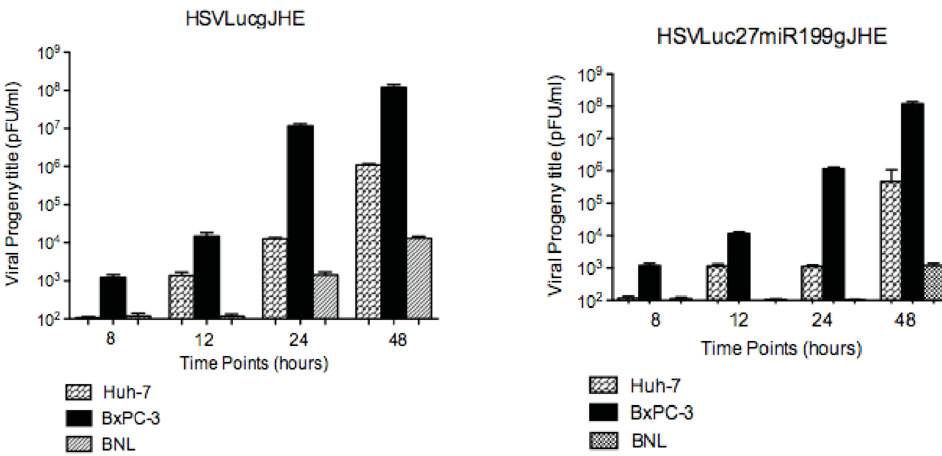


Figure 10. Evaluation of viral progeny production from the different cell lines, infected with HSVLucgHJE and HSVLuc*27tmiR199*gJHE.

To evaluate the viral replication, the cell lines were infected with both HSV-modified vectors at MOI 0,05 and the samples collected at different time points post-infection.

We have observed, as shown in figure 10, production of viral particles in the BxPC-3 and Huh-7 cells infected with both viruses; on the contrary there was almost no replication in BNL cells infected with the vectors.

In figure 11 is shown the cytotoxic effect from both HSVLucgHJE and HSVLuc*27tmiR199*gJHE viruses. In infected Huh-7 the survival of the cells was MOI dose dependent for both viruses, whereas in infected BxPC-3 cells the toxicity was evident also at low MOI for HSVLucgHJE and HSVLuc*27tmiR199*gJHE viruses. The control virus has shown to be cytotoxic in BNL infected cells while in the cells infected with HSVLuc*27tmiR199*gJHE there was not cytotoxic effect even at high MOI of infection.

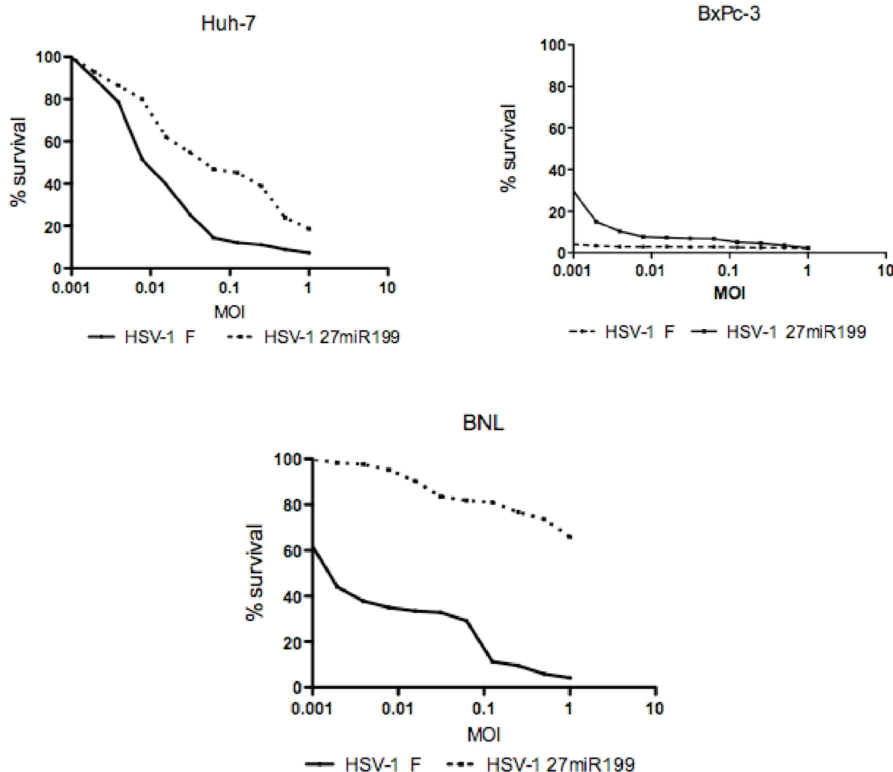


Figure 11. Cell viability on Huh7, BxPC-3 and BNL infected cell performed with Crystal-violet assay after 5 days post infection.

The explanation these data was elucidated when the above-mentioned cell lines were transfected with the pGL3LucmiR-199 plasmid where the luciferase expression is regulated by the presence of miR-199 in the cells. In the figure 12 is evident that Huh7 and BxPC-3 were not expressing miR-199 while BNL was expressing miR-199 that regulates the ICP27 expression in the HSVLuc*27tmiR199*gJHE recombinant vector and the viral replication was blocked in this last cells; HepG2 and Vero cell lines were used as controls for basal expression of miR-199.

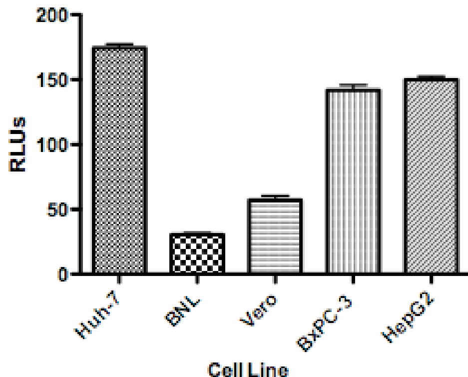


Figure 12. Expression of luciferase miR-199-dipendent in transfected cell lines with pGL3LucmiR199 plasmid.

Evaluation of anti tumor effect in nude mice

The original attempt in this thesis was to evaluate the antitumor effect in two different kinds of tumor mouse models: a syngenic tumor model in immunocompetent Balb/c mice and, in parallel, a xenograft tumor model in nude mice. *In vitro* previous data have demonstrated that BNL were expressing miR-199 (Fig.12) and were not susceptible to the oncolytic virotherapy of our targeted HSVLuc*27tmiR199*gJHE vector (Fig.11) for this reason there was not rational motivation to test these vectors in, *in vivo*, BNL tumor model in Balb/c mice. We have concentrated our efforts in BxPC-3 cells *in vivo*. The preference of these cells in place of Huh-7 was due to the fact that *in vivo* the BxPC-3 cells were growing faster and were susceptible to both HSV vectors also at low MOI. For the tumor development $2x10^6$ BxPC-3 cells were inoculated in the left side of 23 *nu/nu* female mice. After 10 days the mice have developed a tumour of approximately 200-300 mm^3 and the animals were treated by intra-tumor injection with saline solution as control group and with HSVLuc*27tmiR199*gJHE vector, the exclusion of a group with HSVLucgJHE infection was due to the fact that in these cells the control vector have shown the same behaviour of the target vector in BxPC-3 cells *in vitro,* but *in vivo* have demonstrated to be toxic for healthy tissues.

Mouse	Saline Solution	HSVLuc27tmiR199gJHE (single dose)	HSVLuc27tmiR199gJHE (three doses)
Nude (7 mice)	100 ml		
Nude (8 mice)		$2x10^6$	
Nude (8 mice)			$2x10^6$

Table 4. HSVLuc*27tmiR199*gJHE and saline solution administration in nude mice.

As indicated in table 4 one group of nude mice has received a single dose of $2x10^6$ and another group has received three doses with the same virus quantity every 48 hours. As indicated in figure 13 panel A and B, in the group that has received three doses the anti-tumor effect was very strong, but the mice were died for virus toxicity (8/8 animals died), while in the group that have received a single dose was also evident the anti-tumor effect but with a lower toxicity. All animals in the control group were sacrificed between days 40 and 50 due to the tumour size.

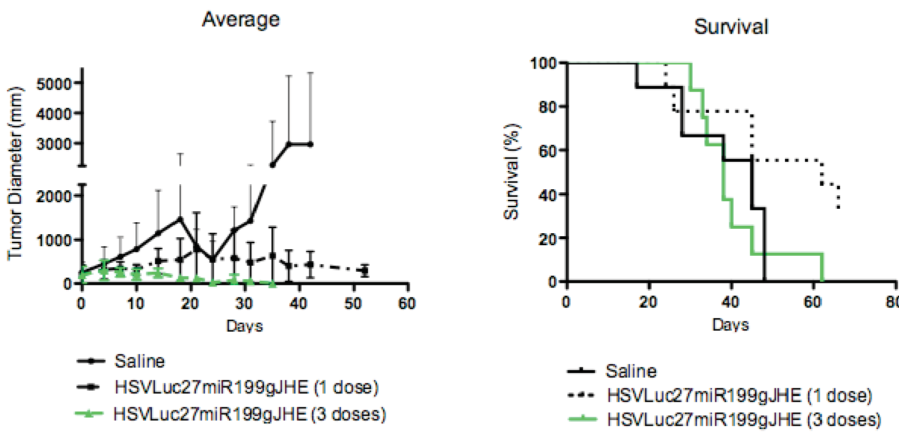


Figure 13 A and B panel. The average (A) and survival (B) of nude mice treated with HSVLuc*27tmiR199*gJHE vector and saline solution.

VECTOR ENHANCEMENT

The objective of the second part of this thesis was to improve the anti-tumor activity of the targeted oncolytic HSV vectors that has been developed in the first part of this work. To this end the HSVLuc*27tmiR199*gJHE vector was engineered to deliver the transgene encoding the IL-12 regulatory cytokine in order to induce local or systemic effects and contribute in the elimination of tumor cells that have escaped to the direct killing through viral oncolysis.

The main goal of this, still on going, part was to find the optimal balance between this transgenic anti-tumor function, without decreasing virus replication and increasing the vector safety and efficacy.

We have constructed two vectors, based on the HSVLuc*27tmiR199*gJHE, that are expressing IL-12. The cytokine was cloned under the control of the tumour-specific hypoxia-dependent promoter (9XHRE) in order to express IL-12 only in the tumour cells, but not in normal cells where this promoter is not active, or under the control of ubiquitous human cytomegalovirus promoter (HCMV) as control vector.

Construction of pgJ1HCMVmIL-12 and pgJ1.9XHREmIL-12 plasmids

The 2023 bp of EcoRV – SwaI fragment from pORFmIL-12, corresponding to mIL-12 coding sequence and SV40 poly(A), was cloned into pgJ1HE, previously digested with PmeI under HCMV promoter. The ClaI fragment corresponding to 655 bp of 9XHRE promoter from pGL3.9XHRE (kindly given by Alberto Epstein) was cloned into pORFmIL-12. The resulting plasmid pORF.9XHREmIL-12 was, subsequently excised with a triple digestion in SnaBI-NotI-SwaI to obtain a cassette with 9XHRE promoter and mIL-12 gene. The 2687 bp fragment was cloned into pgJ1HE in place of EGFP (Fig. 14).

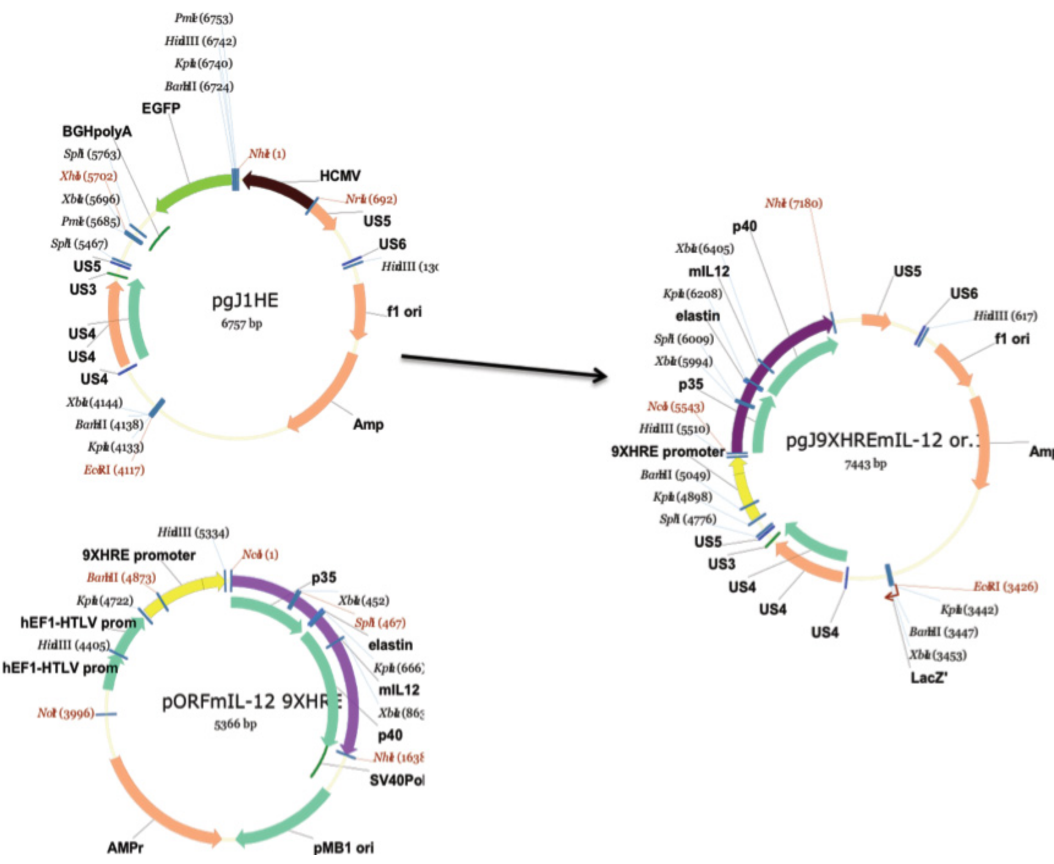
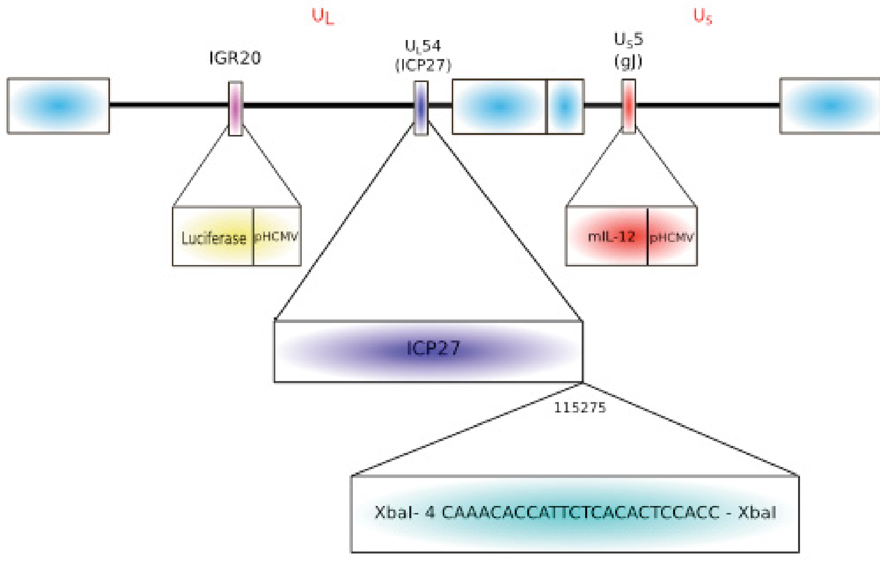


Figure 14. Plasmid map indicates the construction of pgJ1.9XHREmIL-12

Construction of HSVLuc27tmiR199gJHmIL12 and HSVLuc27tmiR199gJ9XHREmIL12 recombinant vectors

HSVLuc*27tmiR199*HCMVmIL-12 vector was constructed starting from HSVLuc*27tmiR199*gJHE vector in which HCMVmIL-12 was inserted, in the Us5 HSV locus, by homologous recombination in place of HCMV-EGFP using the standard calcium phosphate. Vero cells were co-transfected with 5 mg of HSVLuc*27tmiR199*gJHE DNA and 1 mg of the pgJ1HCMVmIL-12 recombinant plasmid (see Material and Methods). The same procedure was performed with HSVLuc*27tmiR199*gJHE DNA and pgJ1.9XHREmIL-12 recombinant plasmid. The two viral DNA were isolated and characterized by “clear plaque” screening with fluorescent microscope. The recombinant viruses were purified through three rounds of limiting dilution and the presence of the transgene in the appropriate locus was verified by Southern blot analysis.

The fluorescence microscope analysis and the titration of viral progeny have demonstrated that these new vectors (data not show) have the same characteristics of the parental HSVLuc*27tmiR199*gJHE.



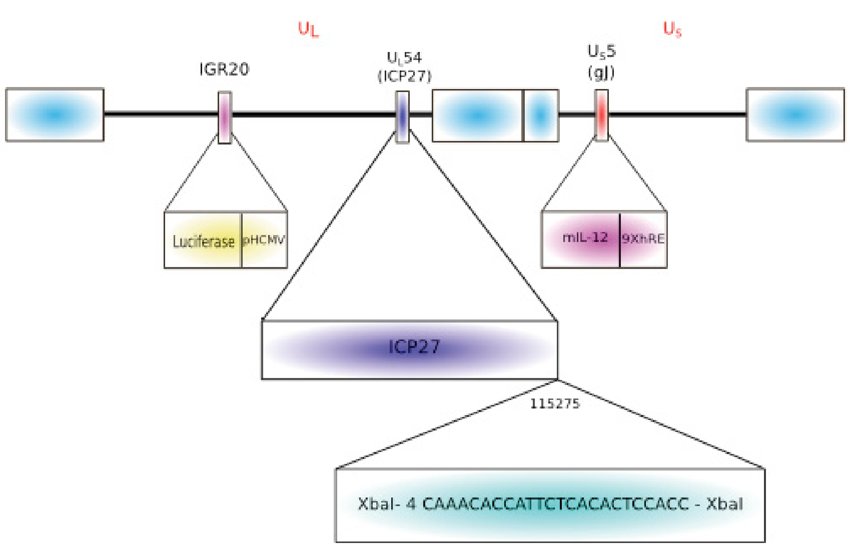


Figure 15. Schematic representation of HSVLuc*27tmiR199*gJHCMVmIL-12 and HSVLuc*27tmiR199*gJ9XHREm-IL-12.

HSV-modified vectors expressed high levels of mIL-12, without affect the viral infection

HSVLuc*27tmiR199*gJ9XHREmIL-12 vector was designed to increase the effectiveness of the virus only into the hypoxic tumor area, without allowing the cytokine expression in non-hypoxic areas (non tumor tissues) while the HSVLuc*27tmiR199*gJHCMVmIL-12 was planned as control vector for mIL-12 expression.

To evaluate the expression level of mIL-12 *in vitro*, $1x10^6$ BxPC-3 cells were infected with HSV-armed mIL-12 vectors at MOI 1 and the samples were recollected 24 hours post-infection and analysed by ELISA mIL-12 (p70) kit (see Material and Methods).

As indicated in figure 16, both vectors were able to express high level of mIL-12 and the lower level detected in samples infected with HSVLuc*27tmiR199*gJ9XhREmIL-12, was due to the impossibility to carrying out the experiment under hypoxia-conditions.

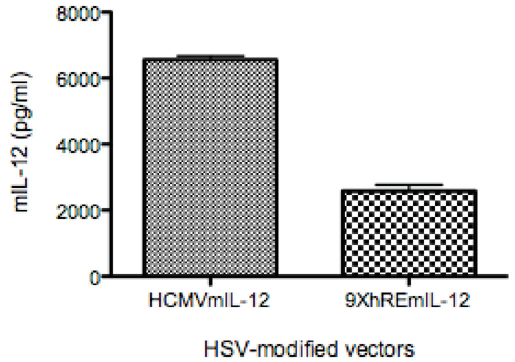


Figure 16. Concentration of mIL-12 in samples infected with HSV-armed mIL-12 vectors.

Evaluation of anti-tumor effect of HSV-armed mIL-12 vectors in vivo

The HSV-mIL12 armed vectors were constructed to increase the anti-tumor activity in syngenic animal models, where the therapeutic role of IL-12 is very important. From previously experiments, BNL cells did not have shown to be responsive to the HSVLuc*27tmiR199*gJHE vector and since we did not have yet the appropriate tumor model for immunocompetent mice we have decided to evaluate and /or to confirm the oncolytic effect of these new vectors in *nude* mice.

16 *nu/nu* female mice were injected subcutaneously with $2x10^6$ BxPC-3 cells into the left side flank. After 10 days all animals have developed a palpable tumour mass, approximately 200-400 mm, and they were divided in three groups. One group of five animals was treated with intra-tumour injection of saline solution as control group; two groups of five and six animals were inoculated intra-tumour with $2x10^5$ infection units (i.u.) respectively of HSVLuc*27tmiR199*gJ9XHREmIL-12 or HSVLuc*27tmiR199*gJHCMVmIL-12 viral vectors. They were followed for 60 days with IVIS scan to evaluate the biodistribution (data not show), viral replication, viral reactivation and anti-tumor activity. As is shown in figure 17, in both groups with the viral vectors there was the same anti-tumour effect, while all animals in the control group were sacrificed due to the tumor size.

The figure 17 indicates the per cent of animal survival: in the HSVLuc*27tmiR199*gJHCMVmIL-12 group, two animals died of lung metastasis and two were sacrificed for treatment ineffectiveness and virus reactivation, respectively; in the HSVLucgJ9XHREmIL-12 group no animal has died due to the virus toxicity.

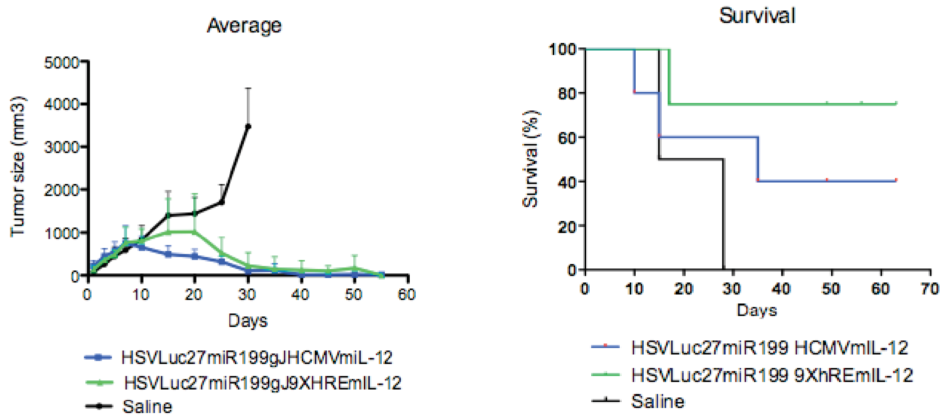


Figure 17. Panels A and B. The average (A) and survival (B) of nude mice treated with HSV-modified vectors expressing mIL-12. The graphic shows the tumour progression from the day of tumor cell inoculation.

Discussion

Discussion

Gene therapy is a form of molecular medicine that promises to provide new treatment for a large number of inherited and acquired human diseases, when and where the conventional clinical procedure are less effective. Actually 64,7% of all gene therapy clinical trials is aim at the treatment of various type of cancer. There is a panel of different gene therapeutic approaches, which either encompass to abrogate the expression of tumor growth promoting genes, or to restore the "normal" status of genes, which are mutated, down-regulated or lost in tumor cells. Other strategies include inhibition of tumor neovessel formation, induction of apoptosis, or stimulation of the immune system.

Oncolytic viruses are emerging as promising options for treatment of cancer, in particular when are used in combination with standard therapies or are adapted as vectors to deliver therapeutic genes [13]. To date oncolytic virotherapy has shown to be safe, and has generated clinical responses in tumors that are resistant to chemotherapy or radiotherapy.

Different viruses are studied and currently used as vectors: derived from oncoretrovirus, lentivirus, adenovirus, adeno-associated virus (AAV) and herpes simplex virus (HSV). In particular, herpes simplex virus-1 (HSV-1) has proven to be an excellent viral oncolytic vector to treat different types of cancer. The replicative and oncolytic nature of these viruses permit *in situ* viral multiplication and spread of the viral infection throughout the tumor mass causing lytic cell death. Several phase I and phase II clinical trials have shown, that HSV-1 viral therapy was well tolerated by patients and in some cases showed considerable efficacy.

We have concentrated the efforts in targeting virus multiplication to specific tumor cells. Instead of engineering improved attenuated virus vectors based on the deletion of viral determinants of virulence, we are optimizing the strict targeting of non-attenuated HSV-1 to a particular cancer tissue, which simultaneously improve efficacy. Through two different ways by the enhancement of their spreading and killing potencies within the tumor mass, and safety by ensure minimum or no local or systemic toxicity towards healthy tissues.

The key concept of this project has been to target virus multiplication to hepatocellular carcinoma cells. The hepatocellular carcinoma (HCC) is the sixth most common type of malignant cancer in adults and the third leading cause of cancer death in the world. Different etiological factors are associated with HCC, such as chronic inflammation, infection by HBV and HCV viruses, liver steatosis and cirrhotic conditions [100]. This last

condition, in particular, makes no effective the classical treatment because their high toxicity in cirrhotic patients. In this regard oncolytic virotherapy offers a promising therapeutic option to treating HCC. In the last years, several studies revealed that the expression of miRNAs is deregulated in human HCC in comparison with matched non-neoplastic tissue. Members of the miR-199 family emerged in several studies as frequently down-regulated in HCC, ovarian, lung cancer and pancreatic. Based on these observations, we have developed a novel strategy for targeting HCC by taking advantage of the differential miR-199 expression level between normal and cancer cells.

In this study we have constructed a miR199-controlled HSV1-based oncolytic vector, designated HSVLuc*27tmiR199*, to test the ability of miR-199 to regulate infected cells protein (ICP) 27 gene expression. ICP27 is an HSV-1 immediate-early protein (IE) that regulates the synthesis of early (E) and late (L) viral proteins. Inhibition of ICP27 expression blocks progression of the virus cycle leading to an abortive infection with no yield of progeny virus. HSVLuc-27*tmiR199* vector is a derivative of HSVLuc, previously constructed in our laboratory, containing four copies of the miR-199 target site between the stop codon of ICP27 gene and the poly(A). Due to the complementarities of these target sites to miR-199, we have shown that the miRNA can mediates the destruction of ICP27 mRNA in normal cells, inhibiting viral replication. We have compared the replication capacity of HSVLuc27*tmiR199* in a panel of human HCC-derived cancer cell lines (HepG2, Hep3B, Huh-7), HepG2 engineered cells constitutively expressing miR-199 (HepG2miR199) and normal cells (HUVEC, primary fibroblasts and primary hepatocytes,). From these, *in vitro,* experiments we have demonstrated that the expression of miR-199, in the cells, drastically reduces the viral replication when target sequences are present in the HSV genome. The ICP27 mRNA expression and, as consequence, the ICP27 protein expression and viral replication, are regulated in a dose-dependent manner. Therefore, miR-199 expressed by HepG2miR199 cells in infections with high MOI of target miR-virus have shown no effect in halt the virus replication, suggesting that the regulation of gene expression and viral replication are directly proportional to the amount of miR-199 expressed by the cells. When the infection is up to 0,1 MOI the miR-199 is not sufficient to block all the ICP27 expression leading to a small amount of viral protein that is sufficient to initiate lytic viral replication. *In vivo* we have analyzed the biodistribution and viral replication, on *nude* and Balb/c female mice, using as *in vivo* marker the luciferase expression present in the viral genome of both recombinant viruses, HSVLucgJHE and

HSVLuc27*tmiR199*gJHE. The luciferase expression has indicated that the miR-regulated vector was not able to replicate in normal liver tissue compared to control vector. The HSVLucgJHE, indeed, has shown a progressive increase in the luciferase signal not only in the abdominal area but also in other parts of the mice body. To evaluate the anti-tumor activity of these HSV-vectors we performed a several *in vitro* and *in vivo* experiments to find a liver murine tumor model, but unfortunately we did not found an appropriate model for immunocompetent mice and, for this limitation, we have decide to evaluate and /or confirm the oncolytic effects of these vectors in *nude* mouse model of human pancreatic adenocarcinoma (BxPC-3 cells). The choice of BxPC-3 cells in place of human HCC cells, Huh-7, was due to the fact that *in vivo* the BxPC-3 were growing faster and were equally susceptible to both HSVLucgJHE and HSVLuc27*tmiR199*gJHE vectors. From previously experiments of toxicity we noted that the injected animals have tolerated higher doses of the HSVLuc27*tmiR199*gJHE vector, compared to the control vector. For this reason we have evaluated the HSVLuc27*tmiR199*gJHE virus toxicity and anti-tumor effect by the administration of high dose of this recombinant into the tumor mass in order to obtain the maximum oncolytic effect and to gain in safety. The animals have received the virus in a single dose or in three separated doses, with the same amount of virus. We have obtained a strong anti-tumor effect with both types of administration, but the animals that have received the three doses have presented a high toxicity. The explanation can be due to the fact that, as have been demonstrated *in vitro*, the IC27 expression is regulated by a dose-dependent manner and, also *in vivo* the miR-199 in the healthy tissue was not sufficient to block all the ICP27 expression. The same experiment has been performed using a 5-fold lower dose of the same virus to assess whether a lower dose of virus was able to give the same anti-tumor effect was maintained. We have observed that the anti-tumor effect was very strong without severe toxicity, suggesting that our HSV-modified vector is able to destroy the tumor cells even at lower doses that can be well tolerated by the animals (data not show).

The objective of the second part of this thesis was to enhance the anti-tumor activity of the targeted oncolytic HSV vectors by introducing a transgenic function, such as murine IL-12. The HSV-based vectors have been engineered to deliver the transgene encoding the regulatory cytokine that can induce local or systemic effects, to contribute in the elimination of tumor cells that have escaped to the direct killing through viral oncolysis. We have constructed two vectors based on the HSVLuc27*tmiR199*gJHE that are expressing

mIL-12, one under the control of the tumor-specific promoter 9XHRE (HSVLuc27*tmiR*9XHREmIL-12), in order to express the cytokine only in the tumor-hypoxic cells, and the other under the control of human cytomegalovirus promoter (HSVLuc27*tmiR199*HmIL-12) as control vector. *In vitro* we have demonstrated the significant mIL-12 level expression in the cells infected with HSV-vectors armed with mIL-12. *In vivo*, a xenograft pancreatic animal model had been treated with a significant lower dose of these new viral vectors ($2x10^5$ i.u.). Again we can observed an anti-tumor effect with both viruses but, very interesting, the animal inoculated with HSVLuc27*tmiR199*HmIL-12 have presented a major toxicity and skin lesions, compared with animals treated with hypoxia-regulated mIL-12 vector, suggesting a major expression of this vector of mIL-12 only into the tumor area, in accord to the hypoxia-dependent promoter. From these date we can hypothesized that the our target HSVLuc27*tmiR*9XHREmIL-12 vector is capable to kill and replicate selectively in tumor cells, in which the levels of miR-199 are very low and the presence of mIL-12 under 9XHRE promoter into HSV genome conferred a select enhancer anti-tumor activity without giving toxic effects on surrounding tissues.

In conclusion, the possibility of developing an anticancer therapy whose activity increases with time, while retaining tumor-specificity and expressing multiple anti-tumor activities is a new and still uncharted area of cancer therapy and, in this context, the administration of HSV-1 oncolytic vectors shows a considerable promise to provide new tools for the treatments of tumors that are resistant to traditional therapies, such as chemotherapy or radiotherapy, improving life expectancy, quality of life, health and safety.

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