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CICLO XXIV

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Regulation of adenovirus replication by miR-199 confers a selective oncolytic activity in hepatocellular carcinoma

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Abstract

Oncolytic virotherapy represents a growing field of experimental cancer therapy. For safe and effective virotherapy, restricted tissue expression and replication of the virus is desirable. Various methods have been developed to achieve such restricted expression. They included the engineering of viral genomes through the insertion of tissue-specific promoters or genes encoding for tissue specific binding proteins. Here, we employed a new approach based on the use of microRNAs (miRNAs) to achieve tumor-specific viral expression and replication. miRNAs are approximately 22-nucleotide (nt)- long non-coding RNAs that are able to bind the 3' untranslated regions (UTRs) of homologous target mRNAs and causing either their degradation or translation inhibition. Since miRNA are differentially expressed in cancer versus normal cells, it is theoretically possible to make virus expression restricted to cancer cells in a miRNA-dependent manner.

Several studies have shown that miR-199 is significantly down-regulated in primary hepatocellular carcinoma (HCC) tissue and HCC cell lines. With this notion in mind, we developed a conditionally replication-competent oncolytic adenovirus, Ad-199T, by introducing four copies of miR-199 target sites within the 3′ UTR of the E1A gene, which is essential for adenovirus replication.

In vitro studies of the properties of Ad-199T virus revealed that E1A expression was indeed tightly regulated both at RNA and protein levels depending upon the expression of miR-199. Consequently, Ad-199T could replicate in the HCC derived cells HepG2, negative for miR-199 expression, while its replication was strictly controlled in HepG2-199 cells, which were engineered to express high level of miR-199. A replication-competent miRNA independent Ad-Control was also generated,. Thus, these *in vitro* studies proved that cytotoxicity of Ad-199T was effective in HCC derived cells, which lacks expression of miR-199, and could be successfully controlled in cells that express miR-199 at high level.

To assess *in vivo* properties of Ad-199T, we tested an orthotopic tumor model. HepG2 cells were implanted in the liver of newborn B6D2 mice. The cells could survive at least one week in this environment, enough for testing *in vivo* properties of Ad-199T. These studies revealed that intrahepatic delivery of Ad-199T led to virus replication in HepG2 derived xenograft tumors and a faster removal of cancer cells. Conversely, Ad-199T replication was not detected in normal, miR-199 positive, liver parenchyma.

These results demonstrate that Ad-199T is a conditionally replicative adenovirus (CRAd) miR-199 dependent, with antitumor activity *in vivo*. This system allows replication of the oncolytic virus in HCC cells and, at the same time, tightly control replication in normal liver tissues, thus avoiding or reducing hepatotoxicity.

Riassunto

L'uso di virus oncolitici per il trattamento del cancro rappresenta un settore sperimentale in continua crescita. Ai fini di garantire l'efficacia e la sicurezza della terapia con virus oncolitici è necessaria un'espressione e una replicazione del virus limitata ai tessuti bersaglio di terapia. Sono stati sviluppati diversi metodi che garantiscono l'espressione limitata desiderata, tra cui manipolazione dei genomi virali attraverso l'inserimento di promotori tessuto-specifici o di geni che codificano per recettori tessuto-specifici. In questo studio abbiamo seguito un nuovo approccio basato sull'uso di microRNA (miRNA) per ottenere l'espressione e la replicazione virale tumore-specifica. I miRNA sono RNA non codificanti lunghi approssimativamente 22-nucleotidi (nt) in grado di legare le regioni 3' non tradotte (UTR) di mRNA bersaglio omologhi, provocandone la degradazione o inibendone la traduzione. Poiché l'espressione dei miRNA nelle cellule tumorali è diversa da quella in cellule normali, teoricamente è possibile limitare l'espressione del virus alle cellule tumorali in maniera miRNA- dipendente.

Diversi studi hanno dimostrato che il miR-199 è significativamente sotto-espresso nei tumori primari e nelle linee cellulari derivate da epatocarcinoma (HCC). Partendo da tale presupposto abbiamo sviluppato un adenovirus oncolitico replicativo condizionale, Ad-199T, introducendo quattro copie di siti bersaglio del miR-199 nella regione 3′ UTR del gene E1A, essenziale alla replicazione virale.

Studi *in vitro* delle proprietà del virus Ad-199T hanno mostrato che l'espressione di E1A era effettivamente strettamente regolata, sia a livello di RNA che a livello di proteina, a seconda dell'espressione del miR-199. Pertanto la replicazione di Ad-199T risultava possibile nelle cellule HepG2 derivate da HCC, negative per l'espressione del miR-199, mentre la replicazione era strettamente controllata nelle cellule HepG2-199, manipolate per esprime livelli elevate di miR-199. È stato inoltre generato il virus Ad-Control, competente per la replicazione in maniera indipendente dal miRNA. Pertanto, gli studi *in vitro* hanno dimostrato che la citotossicità dell' Ad-199T è efficace nelle cellule derivate da HCC, che non hanno espressione del miR-199, e poteva essere controllata in cellule che presentano un alto livello di espressione del miR-199.

Al fine di stabilire le proprietà *in vivo* dell' Ad-199T, abbiamo testato un modello di tumore con sede epatica. Cellule HepG2 sono state impiantate nel fegato di topi neonati B6D2. Le cellule sono sopravvissute almeno una settimana in questo ambiente, cioè un lasso di tempo sufficiente per

testare *in vivo* le proprietà dell' Ad-199T. Questi studi hanno dimostrato che l'iniezione intraepatica di Ad-199T portava alla replicazione del virus nei tumori trapiantati derivati da HepG2 e consentiva la rapida rimozione delle cellule tumorali. Per contro, non è stata riscontrata replicazione di Ad-199T in parenchima epatico normale, positivo al miR-199.

I risultati provano quindi che Ad-199T è un adenovirus replicativo condizionale (CRAd), dipendente da miR-199, con attività antitumorale *in vivo*. Questo metodo consente la replicazione del virus oncolitico in cellule HCC, riducendo o evitando allo stesso tempo l'epatotossicità.

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And finally and by no means the least thanks to my father and family for all their support.

DEDICATION

I dedicate this thesis

- *To My Mother ..*

the only person that more happy to my success.
Be proud of your children. May her soul rest in peace in paradise....

- *To my children ..*

Yosif and Ahmed for making

everything worthwhile.

Baha

Contents

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List of abbreviation

<	Less-than sign
µg	Microgram
Ad	Adenovirus
ADP	Adenoviral death protein
AIDS	Acquired Immune Deficiency Syndrome
bp	Base pairs
CAR	Coxsackie- Adenovirus Receptor
cDNAs	Complementary DNAs
cm	Centimeter
CMV	Cytomegalovirus
CPE	Cytopathic effect
CRAds	Conditionally replicating adenoviruses
CsCl	Cesium Chloride
Ct	Threshold cycle
CVA21	Coxsackievirus A21 gene
DDR1	Discoidin Domain Receptor
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
E. coli	Escherichia coli
E1A	Adenoviral E1A gene
E1B	Adenoviral E1B gene
E2	Adenoviral E2 gene
E3	Adenoviral E3 gene
E4	Adenoviral E4 gene
EGF	Epidermal Growth Factor
EGFP	Enhanced Green Fluorescent Protein
EGFR	Epidermal Growth Factor Receptor
ER	Endoplasmic reticulum
gH gene	Viral glycoprotein H gene
h	Hour (s)
HBV	Hepatitis B Virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HepG2	Cells line derived from human HCC
HepLuc	HepG2 derived cells line constitutively expressing Luciferase
HIF-1α	Hypoxia-inducible factor-1 α
HSV-1	Herpes Simplex Virus 1
HSV-Tk	Herpes Simplex tymidine kinase
IFN	Interferon
IMDM	Dulbecco's Modified Iscove's Medium
IRES	Internal ribosomal entry site
ITRs	Inverted terminal repeats
Iva	Intermediate viral gene in adenovirus
IVIS	*In Vivo* Imaging System

IX	Intermediate viral gene in adenovirus
kb	kilobase (s)
Kg	kilogram (s)
L1-L5	Late viral genes in adenovirus
LCSOV	Liver-cancer-specific oncolytic virus
mg	Milligramme (s)
MHC	Major histocompatibility complex
min	Minute(s)
miRNAs	MicroRNAs
ml	Milliliter(s)
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MV	Measles Virus
ng	Nanogram
nt	Nucleotide
OLT	Orthotopic liver transplantation
ORF	Open Reading Frame
pAd DEST	pAd/CMV/V5-Dest
PCR	Polymerase Chain Reaction
PDGFR-beta	Platelet-derived growth factor receptor beta
qRTPCR	quantitative Real-Time PCR
RIPA	Radio Immuno Precipitation Assay
RNA	Ribonucleic acid
rpm	Rounds per minute
s	Second(s)
SD	Standard deviation
SDS	Sodium Dodecyl Sulfate
T199	Target sequence responsive to miR-199
TACE	Transcatheter arterial chemoembolization
TNFα	Tumor necrosis factor α
TP53	Tumor suppressor protein
ul	Microlitre
UTRs	Untranslated regions
VEGFR	Vascular endothelial growth factor receptor
VGF	Vaccinia growth factor
vp	Viral particles
VSV	Vesicular stomatitis virus
wtAd5	Wild type adenovirus serotype 5

1. INTRODUCTION

Cancer is a major cause of death globally. Although treatments have improved significantly in the last decades, conventional chemotherapy or radiotherapy still have limited effects against many forms of cancer, not to mention a plethora of treatment-related side effects. This situation means that novel more effective and less toxic therapeutic strategies are needed. To address these issues, numerous attempts are underway. All these are driven by an improved understanding of the molecular basis of cancer. The identification of tyrosine-kinases activated in cancer led to the development of several targeted drugs, either small molecules or antibodies, that are presently clinically employed as anti-cancer therapeutics.

1.1 Liver Cancer

Hepatocellular carcinoma (HCC) is one of those human cancer where therapy is often ineffective. HCC accounts for 85–90 % of all primary liver cancers. It ranks as the fifth most prevalent malignancy worldwide and the third leading cause of death (1). The development and progression of HCC is typical of a multistage process. The transformation begins in the liver tissue undergoing chronic hepatitis or cirrhosis caused by external stimuli (hepatitis B virus (HBV) or HCV infection, intake of aflatoxin B1, or alcohol abuse), progresses through a series of hyperplastic and dysplastic stages, and ultimately acquires the malignant phenotype with intrahepatic metastasis and distal dissemination (2).

Treatment is tailored on the basis of the extent of tumor burden, liver function, physical status and potential treatment efficacy. In very early and early stage HCC, potential curative treatments are available. They include surgical resection, percutaneous ablation and liver transplantation. However, only about 30-40% of cirrhotic patients enrolled in surveillance programs are eligible for these types of intervention (3) and, even after a curative treatment, the recurrence rate approaches 70% at 5 years. In advanced HCC, treatment options are even more limited: curative treatments are not available and traditional chemotherapy proved to be only marginally effective or even toxic in either adjuvant or neo-adjuvant settings. The introduction of sorafenib, a multikinase inhibitor, in the treatment of advanced HCC produced an average three months increase in overall survival, but was associated with a significant toxicity. The identification of new possible targets for the development of non conventional treatments is still needed and will necessarily take advantage of progresses in the comprehension of the molecular pathogenesis of HCC (4)

1.2 Virotherapy

Virotherapy is an experimental approach to treat cancer. It utilizes genetically engineered viruses for selective infection and killing of tumor cells while leaving normal cells relatively unharmed.

The ability of viruses to kill cancer cells has been recognized for more than a century (5) . They achieve this by a number of mechanisms, including direct lysis, apoptosis, expression of toxic proteins, autophagy and shut-down of protein synthesis, as well as the induction of anti-tumoral immunity. The origin of oncolytic virotherapy comes from rare anecdotal reports of cancer patients who experience a temporary remission after contracting a viral infection. These cases usually involve patients with hematologic malignancies, associated immunosuppression and a subsequent naturally acquired infection such as influenza, chicken pox or measles. The obvious hypothesis followed that optimized intentional viral inoculation could be a cure for cancer.

Clinical trials of several naturally-occurring oncolytic viruses were started back in the 1950s. Early work in the area was greatly affected from a lack of standardization of study protocols (6). As example, clinical trials were conducted using serum from viremic blood donors (7, 8) .In spite of these challenges, some of these early clinical studies established transient responses with only a few mild side effects and suggested the potentiality of oncolytic virotherapy (9, 10).

The renaissance of this field in recent years came from advances in knowledge about the interactions between virus and host at the molecular level, combined with the possibility of genetic engineering of the viruses. Viral oncolysis (also termed virotherapy) has been studied intensively over the past two decades to develop a new strategy for cancer therapy (11) Development of different viruses based on differences in their natural tropism, catalytic properties, pathogenicity, and opportunities for genetic engineering, has been investigated (12, 13) Many different viruses have been investigated for their potential use as virotherapy agents, including influenza (14), Herpes Simplex Virus 1 (HSV-1) (15, 16), Semliki Forest Virus (17), Vesicular Stomatitis Virus (VSV) (18), Adenovirus (19), Vaccinia Virus (20), Coxsackie Virus (21), Measles Virus (22) and Poliovirus (23). The potential of oncolytic virus is no longer limited by the properties of the viruses in nature. Due to the application of the accumulated knowledge in the fields of virology, immunology and cancer, new oncolytic viruses were engineered to overcome limits related to efficacy and safety of these therapeutic agent.

1.2.1 Mechanisms of tumor selectivity

The term 'oncolytic viruses' applies to viruses that are able to replicate specifically in and destroy tumor cells, and this property is either inherent or genetically-engineered. When used as anticancer drugs, viruses must meet strict criteria for safety, efficacy and accessibility to pharmacological study in human subjects. Specificity for cancer tissues is the key to safety, and this goal can be achieved through strategies that take advantage by tumor-specific changes of cancer versus normal cell (**Figure 1.1**).

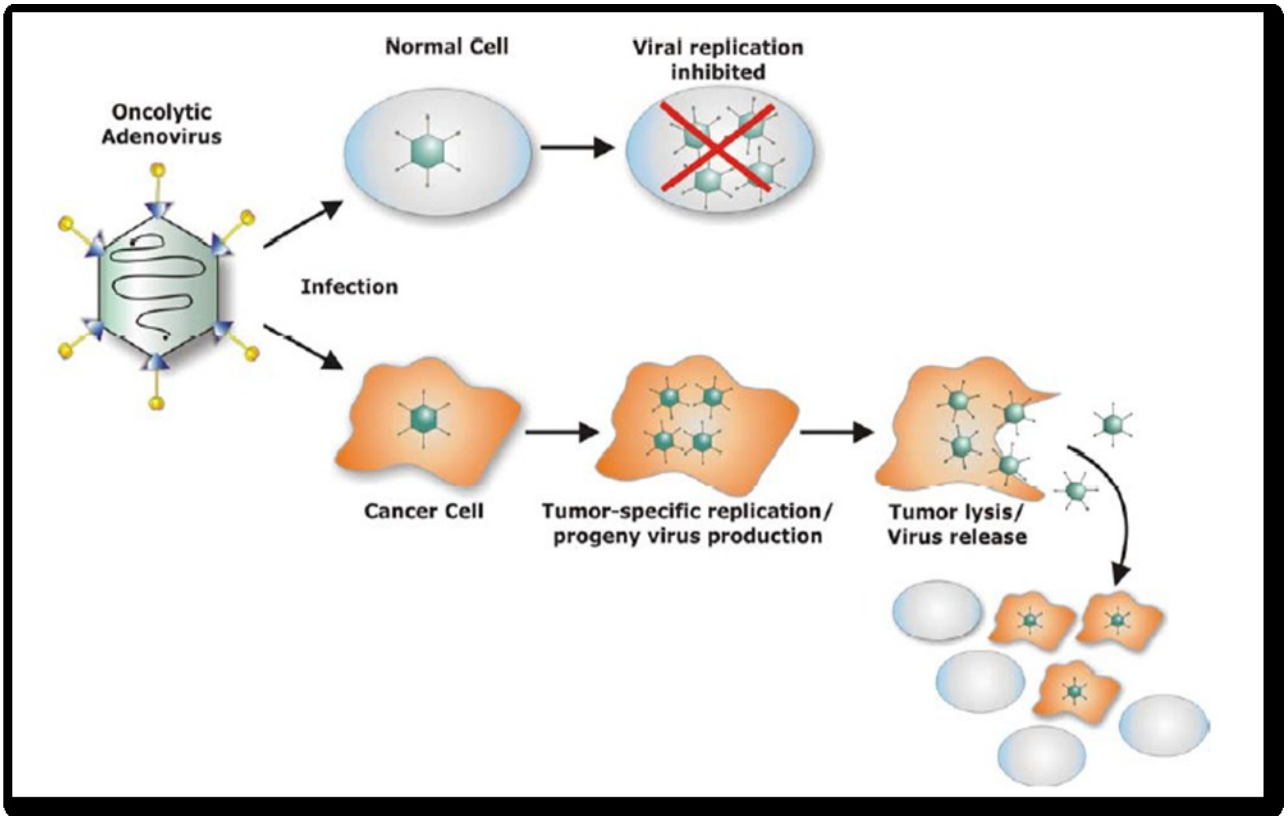


Figure 1.1 Concept of oncolysis. Oncolytic viruses are derived from human viruses via genetic modifications. Such modifications include the mutation or deletion of viral genes, or the insertion of tumor-specific promoters. Oncolytic adenoviruses infect tumor cells, replicate their genome, assemble new viral particles and kill the host tumor cell by lysis, resulting in the release of the progeny viruses. This new virus generation spreads, and starts a new cycle of virus replication and tumor cell killing. Infection of normal cells by oncolytic viruses and/or replication within these cells is impaired. Thus, an ideal oncolytic virus represents an efficient and specific anti-cancer agent.

Inherently tumor-selective viruses can specifically target cancer by exploiting the very same cellular aberrations that occur in these cells, such as surface attachment receptors, activated Ras and Akt, and the defective interferon (IFN) pathway .

Transcriptional and transductional targeting are presently the two main strategies to selectively restrict recombinant adenovirus activity to tumor cells. Transcriptional targeting is mainly achieved by replacing an endogenous viral promoter sequence, e.g., the adenovirus E1A promoter, with a mammalian tumor- or tissue-specific promoter (24, 25) This strategy restricts virus replication to target cells where the promoter is active. Transductional targeting concerns genetic or chemical alteration of capsid proteins for selective infection of tumor cells (24, 25).

Some viruses have been engineered with specific gene deletion; these genes are crucial for the survival of viruses in normal cells but nonessential in cancer cells. Deletion of the gene that encodes thymidine kinase, an enzyme needed for nucleic acid metabolism, results in dependence of viruses such as HSV and Vaccinia Virus on cellular thymidine kinase expression, which is high in proliferating cancer cells but not in normal cells. Vaccinia also produces the vaccinia growth factor (VGF) that binds to and activates the epidermal growth factor receptor (EGFR), creating an environment that supports its replication. It follows that deletion of genes encoding for both thymidine kinase and VGF leads to further selectivity of vaccinia virus in cancers with an activated EGFR-Ras pathway (26).

Another approach in conferring tumor selectivity is to restrict virus replication by its dependence on transcriptional activities that are constitutively activated in tumor cells. This can be achieved by the insertion of a tumor-specific promoter driving the expression of a critical gene (27-33) Others viruses either possess naturally (e.g., Coxsackievirus A21 (34) and Measles Virus (35) or have been designed to have specific tropism based on the expression of cell surface receptors unique to cancer cells (36-42).

Oncolytic adenoviruses (Ad) are genetically engineered Ads which can kill tumor cells by tumor cell-specific replication (43, 44) .

Various types of oncolytic Ads have been developed, and can be mainly classified into 2 groups. One type of oncolytic Ads show tumor-selective replication via deletion of certain genes, such as the E1B-55K gene, which are dispensable for the replication of Ads in tumor cells. The other type of oncolytic Ads possess an E1 gene expression cassette driven by tumor-specific promoters. Various types of tumor-specific promoters are used in oncolytic Ads, including the a-fetoprotein promoter (45) , prostate-specific antigen promoter (46) , osteocalcin promoters (47) and cyclooxygenase-2 promoter (48).

1.3 Adenovirus

Adenoviruses are common opportunistic pathogens rarely associated with severe clinical symptoms in healthy adults (49). Adenoviruses are known to infect humans via the respiratory, the fecal-oral, or the ocular conjunctival routes (50). Illnesses are generally mild in immunocompetent humans, but can spread and cause potentially life-threatening disease in patients with compromised immunity, such as AIDS patients and transplant recipients (50). Adenoviruses belong to the family of Adenoviridae, which is subdivided into four genera (Siadenovirus, Aviadenovirus, Atadenovirus, and Mastadenovirus) (51). All human adenoviruses belong to the genera Mastadenovirus. There are 51 different serotypes of adenoviruses that were originally classified depending on the ability of different animal sera to neutralize them (52). They can be further divided into six different subgroup, A-F, based on their ability to agglutinate erythrocytes of different species and their oncogenicity on rodents. Ad5 from subgroup C is the most widely studied adenovirus, and is the serotype mainly discussed in this thesis.

1.3.1 Adenovirus structure and life cycle

Adenoviruses are non-enveloped viruses 70-90 nm in diameter with an icosahedral capsid. Their genome is linear, double stranded DNA varying between 25-45 kilobases in size with inverted terminal repeats (ITRs) at both termini and a terminal protein attached to the 5´ends (53, 54). The icosahedral capsid is formed by three major proteins, of which the hexon trimers are most abundant (**Figure 1.2**) (55). Each of the twelve vertices of the capsid also contains a pentameric protein, a penton base that is covalently attached to the fiber. The fiber is a trimeric protein that protrudes from the penton base and is a knobbed rod-like structure (56). Other viral proteins such as IIIa, VIII, and IX are also associated with the viral capsid (57). All human adenoviruses have similarities in their fiber architecture. Each has an N-terminal tail, a shaft with repeating sequences, and a C-terminal knob domain with a globular structure (56). The knob domain is mainly responsible for binding the target cellular receptor and its globular structure presents a large surface for lateral and apical binding. The fiber proteins of adenoviruses from different subgroups most distinctively differ in length and ability to bend.

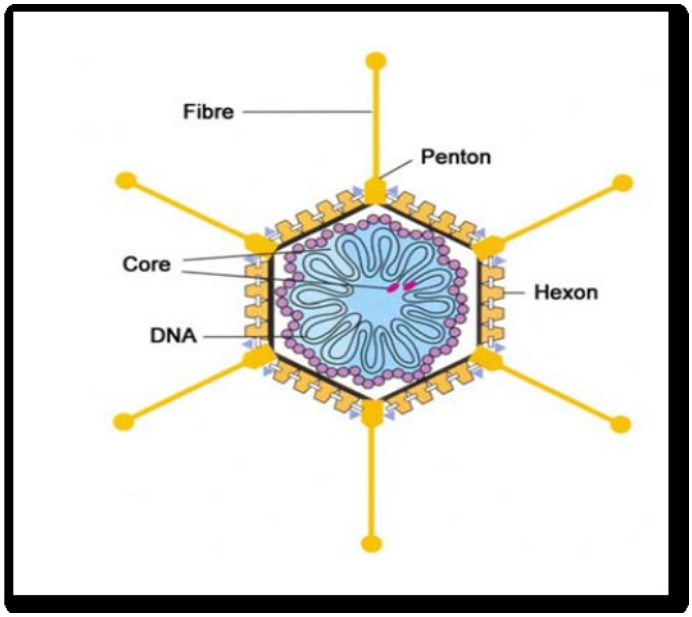


Figure 1.2 Schematic diagram of the Ad5 virion. Adenoviruses are non-enveloped particles of 70-90 nm in diameter with an inner nucleoprotein core. The double stranded DNA is packaged within an icosahedral protein capsid. The major protein of the capsid is the trimeric hexon that constitutes the 20 triangular faces of the icosahedron. Penton capsomeres, formed by the protein of the penton base and fiber, are localized at each of the 12 vertices of the Ad capsid and to which the 12 protruding fiber homotrimers attach (origin: http://biomarker.cdc.go.kr:8080/index.jsp)

Adenovirus trafficking can be characterized by five stages: binding, entry, escape, translocation, and nuclear transport (58). Initially the fiber knob binds a primary receptor with high affinity and once the virus is tethered on the cell surface, low affinity binding to secondary receptors leads to internalization (59). Adenoviruses enter cells via dynamin dependent chlatrin-mediated endocytosis (60). Escape from the endosome into the cytosol occurs within minutes and is dependent on the acidification of the endosome (58). Acidification triggers changes in the adenoviral capsid resulting in the lysis of the endosome membrane. Once in the cytosol, adenoviral capsids translocate towards the nucleus along the microtubules by interacting with cellular molecular motors, such as cytoplasmic dynein (61). Thereafter, the adenoviral genome is transported inside the nucleus via nuclear pores (**Figure 1.3**) (62, 63).

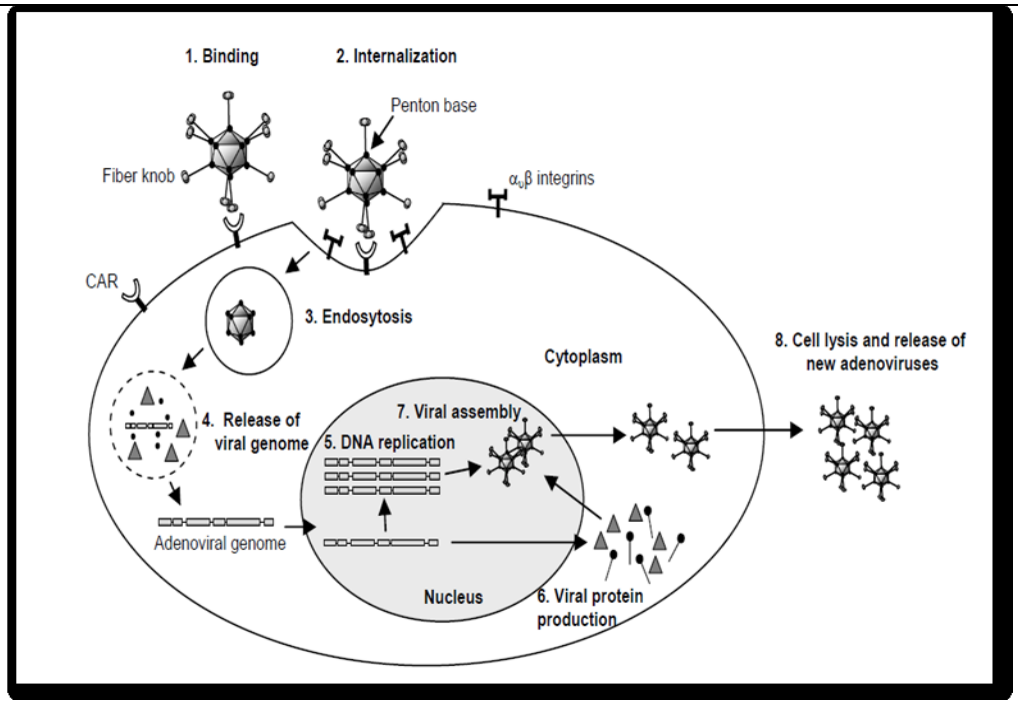


Figure 1.3 Adenoviral replication cycle. Viruses first attach to the coxsackie- adenovirus receptor (CAR) followed by an interaction with cellular integrins resulting in internalization of the virus via receptor- mediated endocytosis. In the endosomes, the viral genome is released from the viral capsid and thereafter transported into the nucleus for DNA replication. Structural viral proteins assemble together with viral genomes in the nucleus followed by cell lysis and release of newly synthesized virions.

1.3.2 Adenovirus transcription and replication

Adenoviruses are dependent on the cellular machinery to replicate the viral genome (64). The adenoviral genome can be divided into immediate early (E1A), early (E1B, E2, E3, E4), intermediate (IX, Iva), and late (L1-L5) genes (**Figure 1.4**) (54). Adenoviral transcription can be described as a two-phase-event, early and late, characterized by the expression of different viral genes and separated by the onset of viral DNA replication (52). The first transcription unit to be expressed is the E1A. The E1A proteins stimulate the transcription of other early genes and modulate the expression of cellular genes involved in the transition into S-phase, making the cell more susceptible to viral DNA replication (65-67). The E1B proteins suppress cell death elicited in response to unregulated cell proliferation signals, including those mediated by E1A (64). The E2 gene products provide the replication machinery for viral gene products. E3 gene products are not essential for virus replication *in vitro*, but are dedicated to the control of various host immune

responses (68). E3-gp19K inhibits the transport of the class 1 major histocompatibility complex (MHC) from the endoplasmic reticulum (ER) to the plasma membrane, thereby preventing the presentation of peptides to T lymphocytes by MHC (69, 70). Other E3 proteins inhibit apoptosis elicited by various cellular proteins such as the tumor necrosis factor α (TNFα) (71). As an exception, E3 derived adenoviral death protein (ADP) functions late in the viral cycle to promote cell death, presumably to aid in the release of the virus after all the replicative functions have been completed (72).

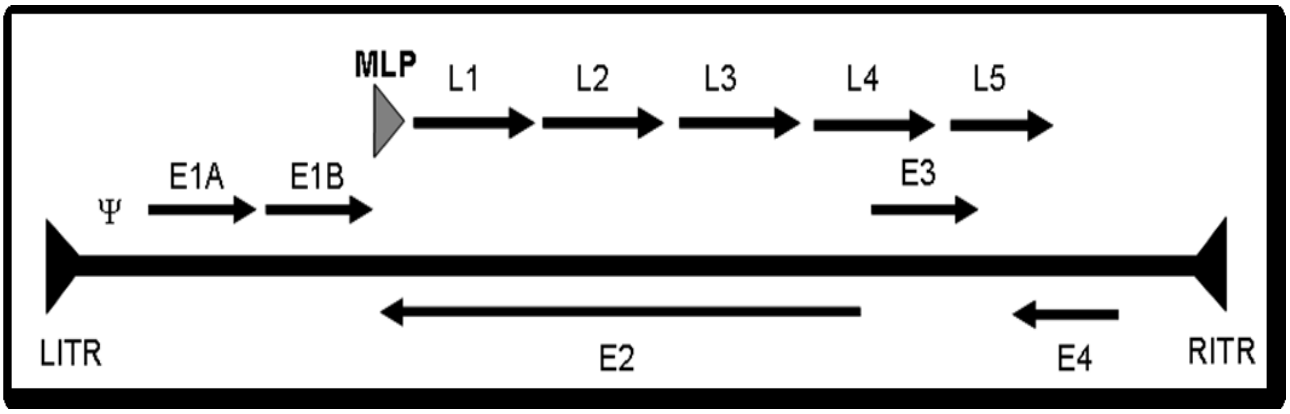


Figure 1.4 Schematic representation of adenovirus genome. The central solid line represents the viral genome. Positions of the left and right ITR (inverted terminal repeats), the packaging sequence (ψ), the early transcription units (E1A, E1B, E2, E3 and E4), and the major late transcription unit (major late promoter (MLP), L1-L5) are shown. Arrows indicate the direction of transcription.

E4 gene products have been implicated in many events that occur as the late program begins. E4 proteins augment viral DNA synthesis and messenger RNA (mRNA) transport, late viral gene expression, shutoff of host protein synthesis, and production of progeny virions (73-76). The late gene transcription leads to the production of viral structural components and the encapsidation and maturation of the viral particles in the nucleus.

Table 1.1 Adenovirus gene products and their functions

Phase	Gene	Products and their function
early	E1A	Inactivates pRB to release E2F - cell cycle deregulation Transactivates viral promoters
	E1B	55kD targets p53 and participate in transport of late viral mRNA 19kD is a Bcl-2 homologue - anti-apoptotic
	E2A	Pre terminal protein (pTP) and DNA polymerase (pol) - DNA replication
	E2B	Single-strand DNA binding protein (DBP) - DNA replication
	E3	gp19kD inhibits MHC I expression 10.4kD/14.5kD (RID complex) inhibits tumor necrosis factor (TNF) apoptosis, internalizes TNF receptor and degrades Fas ligand 14.7kD inhibits TNF apoptosis, stabilized NFκB 11.6kD (ADP) induces cell lysis
	E4	Products: orf1, orf2, orf3, orf4, orf6 and orf6/7 modulate viral mRNA metabolism, promote virus DNA replication and block host protein synthesis
intermediate	IVa2	Initiate the major late promoter (MLP), which regulates late IX genes
	IX	Initiate the major late promoter (MLP), which regulates late IX genes
	VAI	Non-coding RNA that stimulates translation of viral genes and blocks double stranded RNA activated protein kinase R (PKR) during interferon response
	VAII	Non-coding RNA that blocks PKR during interferon response
late	L1-L5	Structural proteins: L1 (IIIa); L2 (penton base, V, VII); L3 (hexon, VI, virus protease); L4 (VIII); L5 (fiber)

1.4 Adenovirus vectors

Adenoviruses represent 25% of vectors used in the clinical trials (77). Some of the reasons behind its popularity are related to the ability to produce the virus at high titer, to infect both dividing and non-dividing cells and to drive a high expression level of the transgene. Moreover, the adenovirus biology is well known and the methods to manipulate the adenoviral genome are relatively easy to perform. The use of adenovirus vectors started with the development of first generation vectors, soon followed by the second and third generation vectors as well as the conditionally replicating adenoviruses (CRAD).

1.4.1 First generation vectors

First generation adenovirus vectors are replication deficient vectors deleted in their E1 genes. They can be produced in cell lines that trans-complement the E1 genes and the most commonly used cells

are the 293 cells (78). These vectors are often deleted also in the E3 region allowing a total insertion size of 6.5-8 kb. Production of the E1-deleted first generation vectors is easy and the recombinant viruses can target a high expression of the transgene in a wide range of cells. In spite of the fact that the efficacy of these vectors is very good *in vitro*, the results have often been disappointing *in vivo*. First generation adenoviruses cannot be used for long term expression of the transgene, since the expression last for a maximum of a few months in immunocompetent individuals (79).

The reason for this is that the expression of viral genes in the E1-deleted vectors leads to immune stimulation. Further drawback of the first generation vectors is the risk of contamination with replication competent adenoviruses (80). Also there is risk that some of the viruses regenerate the E1 region by homologous recombination in the trans-complementing cell line. Beside, the vector can recombine to replication competent viruses if the transduced cell is infected by a wild type adenovirus. For some purposes, where a sustained transgene expression is not needed, the first generation vectors have had some success. Patients with malignant gliomas almost doubled the survival time when administered a first generation adenovirus vector expressing the gene for herpes simplex tymidine kinase (HSV-Tk) followed by administration of ganciclovir as compared to standard treatment with radiation (81). Another approach has been vaccination. First generation vectors have been successful used to induce a potent immune response against pathogens in an effective and rapid manner. For example, one administration of adenovirus vectors expressing the Ebola glycoprotein, induced 100% protection against a lethal dose of Ebola in non-human primates within four weeks, whereas a standard vaccine required at least six months and multiple boosts to reach protection (82). This evidence suggest the possible use of adenovirus-based vaccine in limiting epidemic spread using ring vaccination (82).

1.4.2 Second generation vectors

The second generation vectors have additional genes deleted, for example the E2 region. These are replication-deficient vectors, which are less likely to generate replication competent adenoviruses, but the problems associated with the first generation vectors are still seen *in vivo*. The E1-E4 deleted vectors showed reduced toxicity *in vivo*, produced less viral proteins and had an increased duration of transgene expression (83). However, there are conflicting data regarding the difference in the immune response and the duration of expression compared to the E1 deleted vectors (84).

Some studies indicate that deletion of the E4 region leads to transcriptional silencing of the vector construct (85, 86). The silencing was dependent on the promoter used as well as the tissue type transduced by the vector. The CMV promoter used for transgene expression was silenced in vectors lacking the E4 region, and further analysis showed that retention of the E4 ORF3 was needed to circumvent the gene silencing in murine lung and liver (87, 88).

1.4.3 Third generation vectors

The third generation of adenovirus vectors is the "gutless" adenoviral vectors, named also "high capacity vectors" or "helper dependent vectors". They are free of all viral genes, containing only the viral ITRs and packaging signal (89). This makes it possible to package up to 37 kb in the vector construct. The production of this vectors is more difficult than for the first generation vectors. Since the vector is free of all viral genes, these must be supplemented during the production. This is accomplished by co-infecting the producer cell line (i.e 293 cells) with an E1-deleted helper virus that can produce the proteins needed for particle assembly. Consequently, the gutless virus preparation is often contaminated with E1 deleted replication incompetent helper virus, although most of them can be separated from the gutless virus by equilibrium centrifugation in a CsCl gradient. More optimization of the method by a Cre-loxP system that delete the packaging signal from the helper virus has made it possible to produce gutless preparations with less than 0.1% contamination with helper virus (90). Adenovirus capsid usually contains a genome of about 36 kb. The gutless vector must therefore be of equivalent size to be properly encapsidated. The vector construct contains the transgene expression cassette and a non-coding "stuffer DNA". It has been shown that the nature of the stuffer DNA will influence the expression efficacy from the transgene cassette (91).

These vectors show reduced toxicity and a prolonged gene expression *in vivo*. The gene expression can be sustained for more than a year in baboons (92). Furthermore, approaches that will facilitate long term expression also in dividing cells by introduction of EBNA1 elements into the vector have been appraised (93). Gutless vectors are superior to first generation vectors regarding the duration of gene expression and reduced activation of T-cells cytotoxic after vector transduction. However, the adenovirus capsid can itself be toxic at high concentrations. The adenovirus capsid activates the innate immune response that can induce acute toxicity at high virus concentrations. Systemic injection of 5×10^{12} gutless virus particles/kg into a baboon caused mild and transient acute toxicity whereas 1×10^{13} virus particles/kg induced lethal acute toxicity (94).

1.4.4 Conditionally replicating adenoviruses (CRAds)

Replication incompetent adenovirus have been used clinically for tumor therapy, but the outcome has not been satisfactory. There has been some success for glioma therapy (81), although not all studies have been successful. One problem with the replication incompetent vectors is the poor spread form the site of injection (95). One way to approach this problem is to use conditionally replicating adenoviruses (CRAds). CRAds have been developed to specifically target tumor cells (96, 97).

Among these, there are adenoviruses with modifications that can replicate in p53 deficient tumors. TP53 gene is mutated in about 30% of primary glioblastomas and 65% of secondary glioblastomas. In lower grade gliomas the frequency is even higher (98). For efficient replication of the adenovirus genome, the cell is forced into S-phase by the E1A inactivation of the Rb protein. Forced induction of S-phase may trigger p53 dependent apoptosis and subsequent cessation of adenoviral replication. To prevent this apoptotic response, the E1B 55 kDa protein is necessary to inactivate the action of p53. Adenoviruses with deletions in the E1B 55 kDa have been produced, of which the best known is ONYX-015 (99). Hence, this E1B-deleted virus can replicate in p53-deficient tumor cells, but not in p53-wt normal cells. This virus showed cell lysis and anti-tumor activity also in human glioma cells with or without p53 mutation (100). About 100 patients, mostly with severe cancers, have been treated so far in clinical phases (phases I-III) using the ONYX-015, and the treatment is well-tolerated at doses up to 2×10^{12} particles/patient irrespective of administration route (intratumoral, intraperitoneal, hepatic artery or intravenous administration) (101). The replication in the tumors is transient and is terminated within few days. The anti-tumor effect is synergistic when given in combination with chemotherapy. The combination therapy has shown success in the treatment of head and neck tumors (102).

1.5 Development of targeted adenoviruses

The efficacy and safety of the adenoviral vectors could be improved by targeting the vector to the chosen tissue. Different genetic engineering strategies to restrict viral replication to cancer cells have therefore been employed to minimize toxic side effects.

1.5.1 Transductional targeting

Transductional targeting includes modification of the capsid to direct the vector to a new receptor. Several strategies have been involved , for example the use of bispecific antibodies that bind to the viral capsid and a receptor of choice (e.g. EGF receptor that is often up regulated in tumors). This approach has been shown to ablate the natural tropism for the coxsackie- adenovirus receptor (CAR) and induce a specific binding and uptake through the bispecific antibody (103).

1.5.2 Transcriptional targeting

Transcriptional retargeting involves tissue specific promoters that only allow transcription of the transgene in the tissue of choice. Adenovirus vectors have the capacity to infect a wide range of cell types. For some purposes (e.g. cancer therapy with a toxic gene) it is not favorable to have expression in tissues other than the target organ. The transgene inserted into the vector is accompanied by a promoter of choice, and sometimes it is favorable to also include enhancer regions (104). Environmental signals such as stress, hypoxia or hormones can regulate the promoters response element located within the promoter region. When a cell becomes malignant, the expression of certain genes that normally show low or no expression can become upregulated. This can be exploited in the vector construct by regulating the transgene expression with the activated promoter. Tissue and tumor specific promoters and their use for gene therapy have been reviewed (105). The use of tumor or tissue-specific promoters is actually not so easy in practice. In some cases, the promoter loses specificity when out of its context into vector construct. In addition, these types of promoters are usually too weak to be of therapeutic benefit (106).

To overcome the limits described above, other strategies are currently been developed, based on improvement of vector design (107) and post-transcriptional targeting. The use of microRNA targeting is one of these and is the focus of the work of this thesis.

1.6 MicroRNAs

MicroRNAs are endogenous non-coding RNAs that have been identified as post-transcriptional regulators of gene expression (108). Today, the miRNA registry (miRBase) accounts for 18,226 miRNAs, among which 2154 are human (Release 18 November 2011) (109). Each miRNA can bind

to regions of homology that are often found within the 3' untranslated regions (UTRs) of target mRNAs, resulting in mRNA degradation or translational inhibition. Identification of miRNA targets is crucial to understand of miRNA function and their role in disease pathogenesis.

1.6.1 MicroRNAs and hepatocellular carcinoma

Aberrant expression of miRNAs has been observed in a range of human diseases, which include cancer (110). In human cancer, miRNAs can function as oncogenes or tumor suppressor genes during tumor formation and progression (111).

An increasing number of reports have described the involvement of microRNAs (miRNAs) in HCC development and progression. Differences in miRNA expression between HCC and liver parenchyma could potentially be used for the development of novel therapeutic approaches. In hepatocellular carcinoma (HCC) several deregulated miRNAs have been identified. Some of these have been associated with the clinico-pathological features of HCC, such as metastases, recurrence, and prognosis (112-114). Since the first publication of a miRNA gene expression profile in liver cancer by Murakami *et al*. in 2006, various studies on miRNA expression in HCCs have been reported (**Table 1.2**). While the predisposing risk factors and etiologies of HCCs were heterogeneous, the deregulation of some specific miRNAs was commonly identified in the published studies, suggesting their importance in liver carcinogenesis. Among these, the over-expression of miR-21, miR-221, miR-222, miR-224, miR-301, miR-500 and the under-expression of miR- 122, miR-125a, miR-139, miR-145, miR-150, miR-199a, miR-200b, miR-214, miR-223, miR- 101 were reported by more than one publication. These miRNAs are listed in **Table 1.3**.

Table 1.2 Published miRNA gene expression studies in HCC

Year	Profiling Method	Main conclusions of the studies	Reference
2006	Microarray / Northern blot	Analysis of miRNA expression patterns in HCC and non-tumorous tissues: detection of dysregulated (miR-199a, miR-200a, miR-125a, miR-195 down-regulated; miR-18 and miR-224 up-regulated) miRNAs in HCC versus non-tumor liver tissues	(115)
2006	Microarray / Northern blot	Identification of miRNAs aberrantly expressed in HCCs occurring in male Fisher rats, fed with a diet deficient in folic acid, methionine, and choline. The down-regulation of miR-122 was associated with hepatocarcinogenesis.	(116)
2007	Microarray / Northern blot / qPCR	Demonstration that aberrant expression of miR-21 can contribute to HCC growth, migration, and invasion by modulation of PTEN and PTEN-dependent pathways.	(117)
2007	Microarray / Northern blot /qPCR	Analysis of miRNA expression patterns in human HCC and non-tumorous tissues. Demonstration that miR-122 is down-regulated in HCC and modulates cyclin G1 expression	(118)
2008	Microarray / Northern blot	miRNA expression profiles in 10 pairs of HCC and adjacent non-tumorous tissue from 10 patients without viral hepatitis: identified 40 miRNAs differentially expressed in HCC, whose expression may provide information on pathogenetic mechanisms involved in HCC.	(119)
2008	qPCR	Identification of aberrantly expressed miRNAs in HCC. miR-224, the most significantly up-regulated miRNA, was found to increase apoptotic cell death as well as proliferation and targets apoptosis inhibitor-5 (API-5).	(120)
2008	Microarray / qPCR	Demonstration that miR-221 is up-regulated in HCC compared to non-tumorous tissue; miR-221 can control the expression of the cyclin-dependent kinase inhibitors p27 and p57.	(121)
2008	qPCR	Study the association of MicroRNA Expression in Hepatocellular Carcinomas with Hepatitis Infection, Cirrhosis, and Patient Survival. Results show that Several miRNAs including miR-199a, miR-21, and miR-301 were differentially expressed in the tumor compared with adjacent benign liver.	(122)
2008	qPCR	Study of miRNA expression profile of HCV–associated HCC, a group of miRNAs are aberrantly expressed in primary liver tumors, miR-122, miR-100, and miR-10a were overexpressed whereas miR- 198 and miR-145 were up to 5-fold down-regulated in hepatic tumors compared to normal liver parenchyma.	(123)
2008	qPCR	Identification of distinct miRNA expression signatures according to malignancy, risk factors, and oncogene/tumor suppressor gene alterations.	(113)
2009	qPCR	Study reveals that diverse changes of miRNAs expression occur during liver development and miR-500 is an oncofetal miRNA relevant to the development of HCC.	(124)
2009	microarray/ Northern blot	Demonstrate an important role of miR-101 in the etiology of HCC and its potential use in cancer therapy.	(125)
2010	qPCR	The study identifies a group of miRNAs that discriminate tumors from adjacent cirrhotic liver tissue; it shows that miR-222, which is over-expressed, can induce AKT signaling	(126)
2011	Microarray / Northern blot / qPCR	The study demonstrates the role of miR-139 in Suppression Metastasis and Progression of Hepatocellular Carcinoma by Down-regulating Rho-Kinase 2	(127)

Table 1.3 miRNAs most commonly aberrantly expressed in HCC

miRNA	Chrom. Location	Dysregulation	Reference
miR-26a	3p22	Decreased	(128, 129)
miR-101	1p31.3	Decreased	(125)
miR-122	18q21.3	Decreased	(113, 116-118)
miR-125a	19q13.3	Decreased	(115, 117)
miR-139	7p22.1	Decreased	(120, 122)
miR-150	9p24.3	Decreased	(118, 122)
miR-145	5q32	Decreased	(118, 120, 123)
miR-199a	1q24.3	Decreased	(115, 117, 118, 122)
miR-200b	1p36.33	Decreased	(118, 122)
miR-214	9p24.3	Decreased	(118, 120, 122)
miR-223	Xq12	Decreased	(118, 122)
miR-18	13q31	Increased	(115, 122)
miR-21	17q23.2	Increased	(113, 116, 117, 119, 120, 122, 123)
miR-221	Xp11.3	Increased	(117, 118, 122, 130)
miR-222	Xp11.3	Increased	(113, 117, 118, 126)
miR-224	Xq28.3	Increased	(113, 117, 120)
miR-301	17q23.2	Increased	(120, 122)
miR-500	Xp11.2	Increased	(124)

1.6.2 microRNA 199

Among down-regulated miRNAs, miR-199a-3p is processed from the precursor, hsa-miR-199a, and is down-regulated in several human cancers, including HCC (115, 117, 118, 122, 131, 132). It has been suggested that this miRNA can act as tumor suppressor because it can prevent cell invasion and metastasis by negatively regulating c-MET proto-oncogene and its downstream effector ERK2 (133). Its tumor suppressor function has been more recently strengthened because the mammalian target of rapamycin (mTOR) was also shown to be a target of miR-199a-3p. The inhibitory role of this microRNA in mTOR pathway is able to modulate cell proliferation and the invasion capability (134). The other strand of the same precursor miR-199a, miR-199a-5p, was demonstrated by Shen Q *et al*, to target Discoidin Domain Receptor (DDR1) gene. DDR1 is a tyrosine kinase receptor that is over-expressed in several human cancers (135-139). A down-regulation of miR-199a-5p expression, reported in HCC patients, could promote tumor progression enhanced by DDR1 up-regulation (140) . Interestingly, miR-199 was also involved in inflammatory reactions associated with alcoholic diseases, most of which are mediated by molecules of the hypoxia-associated pathway (141).

In this context, the expression of HIF-1α mRNA (hypoxia-inducible factor-1 α) and its coregulated gene, ET-1, were shown to be targets of miR-199 (142), suggesting a new potential approach in the control of ethanol induced inflammation. MiR-199-a was also identified as having a target sequence in the internal ribosomal entry site (IRES) of HCV viral genome, highly conserved in all HCV genotypes (143). miR-199 was shown to have an anti-viral effect. Murakami *et al*. have shown that the inhibition of miR-199 activity by a specific AMO causes an increase of HCV replication, suggesting that this miRNA could negatively regulate the viral replication and proliferation (144). Recently, scientists found that miR-199a-3p may also play a role in regulation of HBV replication by targeting the HBV S protein coding region, the pre-S coding region and the ORF of HBV polymerase (145).

In summary, miR-199 is involved in various processes that become derailed in liver cancer in consequence of its stable down-modulation in cancer cells. In addition and notably, it also is involved in modulating the effects of HCC risk factors, such as the replication of hepatitis viruses or the effect of alcohol consumption (**Figure 1.5**).

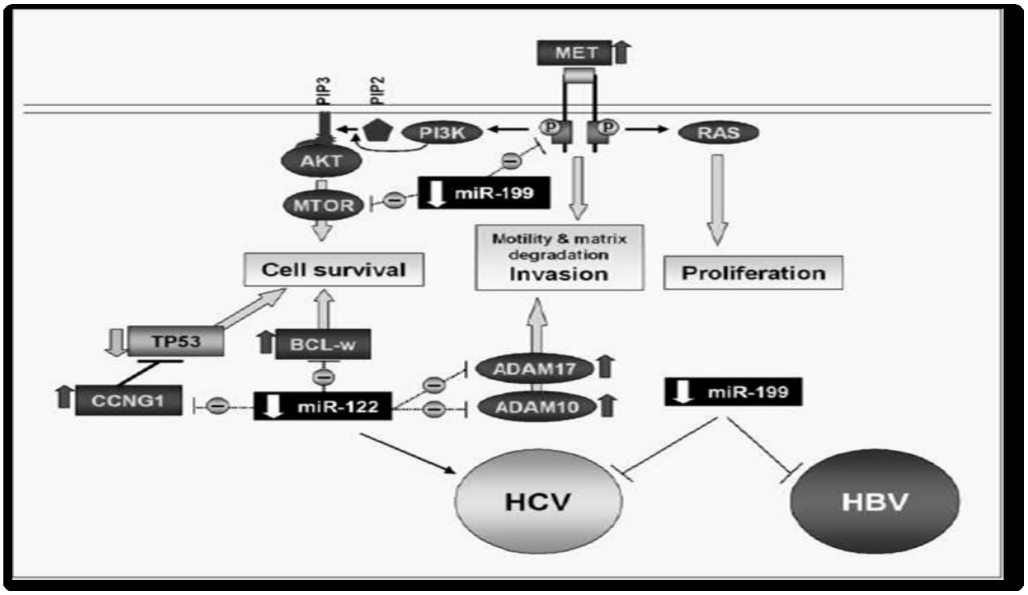


Figure 1.5 Down-regulation of miR-122 and miR-199 in HCC. The diagram shows validated targets for miR-122, that include the genes for cyclin G1 (CCNG1), BCL2-like 2 (BCL-w) and disintegrin and metalloproteinase domain containing protein 10 and 17 (ADAM10 and ADAM17). It also shows targets of miR-199, the gene for mechanistic target of rapamycin (MTOR) and the met proto-oncogene (MET), which encodes for the hepatocyte growth factor tyrosine kinase receptor. The diagram also shows that miR-122 favors the replication of hepatitis C virus (HCV), while miR-199 exerts an inhibitory effect on both hepatitis B (HBV) and HCV viruses, which are one of the principal risk factors for HCC development. The down- regulation of miR-122 and miR-199 in HCC sustains cell survival, proliferation and invasion.

1.6.3 Post-transcriptional targeting of viral vectors' replication

Differential expression of miRNA between cancer and normal tissue can be exploited as a novel strategy for the regulation of viral replication. The structures of attenuated viruses is shown in **Figure 1.6.**

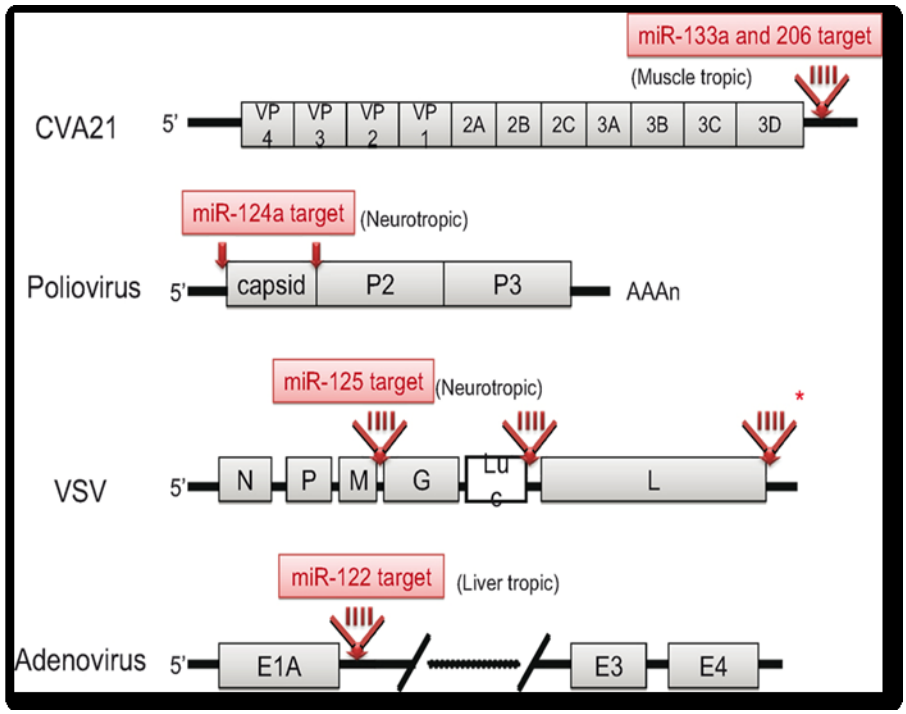


Figure 1.6 Schematic genome structures of attenuated viruses. The localization of miRNA target insertion site in each virus is depicted (red arrow). In the case of Coxsackie virus A21 (CVA21), Vesicular stomatitis virus (VSV) and adenovirus, 4-tandem repeat target sequences (red bars) were inserted. Asterisk indicates the most effective site for attenuation of VSV.

Brown *et al*. (146) were pioneers in developing viral vectors regulated by microRNAs. They developed lentiviral vectors with target sites for mir-142-3p and showed that transgene expression was effectively suppressed in cell types expressing this microRNA. Perez *et al*. successfully attenuated influenza A virus through the incorporation of species-specific, non-avian microRNA (miR-93) targets into nucleoproteins. The result was attenuated viral activity in mice, but not in eggs, as expected (147).Therefore, miRNA-mediated control of viral replication is a promising technology that can be used to safely attenuate virus for the production of live vaccines..

Taking advantage of tissue specificity of miRNAs, Kelly et al. inserted the target sequences of muscle-specific miRNAs (miR-133a and miR-206) in the 3 'UTR of coxsackievirus A21 gene (CVA21), a pathogenic picornavirus that causes lethal myositis and oncolysis in tumor-baring mice (21). The result was tissue-specific attenuation that allowed the virus to replicate well in muscle cells without being pathogenic. Inserting in the poliovirus, target sites for the neuron-specific miR-124a an attenuated poliovirus vaccine lacking any neurovirulence was developed (148).

Similar strategies were also applied to encephalitis caused by vesicular stomatitis virus through the insertion of miR-125 target sequences within the 3 'UTR of the viral polymerase gene (149).

The idea was further developed to evaluate the selectivity of oncolytic viruses by controlling unwanted replication in normal cells (21, 150, 151). This strategy was also used to inhibit adenoviral replication in normal liver cells to reduce the liver toxicity of systemic administration of oncolytic adenoviruses. The incorporation of the liver specific miR-122 target sites in the 3'-untranslated region- of E1A gene resulted in an adenovirus unable to replicate in liver, but full functional in any other cell or tissue (96, 97).

2. AIMS

Cancer is a major public health problem and new therapeutic approaches are needed. In fact, despite progresses in reducing mortality due to improvements in cancer prevention, early detection and treatments, still 6.7 million deaths worldwide are caused by cancer each year (152). Hepatocellular carcinoma (HCC) is the third cause of cancer-related death worldwide, it carries a very poor prognosis and requires more effective therapeutic approaches.

A promising approach is based on oncolytic virotherapy, which relies on the delivery and replication of viral vectors, which mediate the cytolysis of tumor target tissue. Adenoviruses are widely studied as oncolytic agents in cancer virotherapy (5, 153, 154).

Based on the observation that miR-199 is significantly down-regulated in HCC in comparison to normal liver, the aim of this work was: (1) to produce a conditionally replicative adenovirus (CRAD), miR-199-dependent, for the virotherapy of HCC; (2) to assess the miR-199-dependent viral replication *in vitro* and *in vivo* assays; (3) to test the in vivo oncolytic activity of the CRAD vector.

3. MATERIALS AND METHODS

3.1 Plasmids

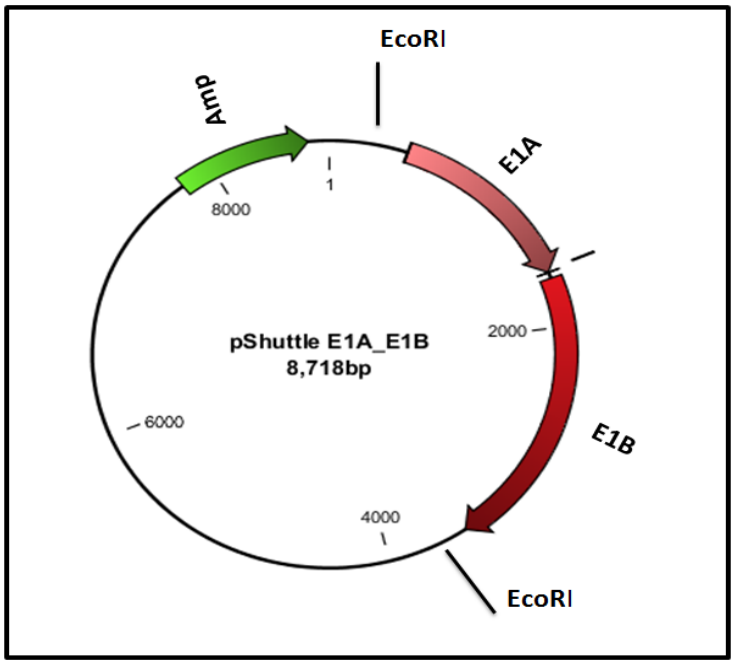


Figure 3.1 Map of **pShuttle E1A_E1B** Vector

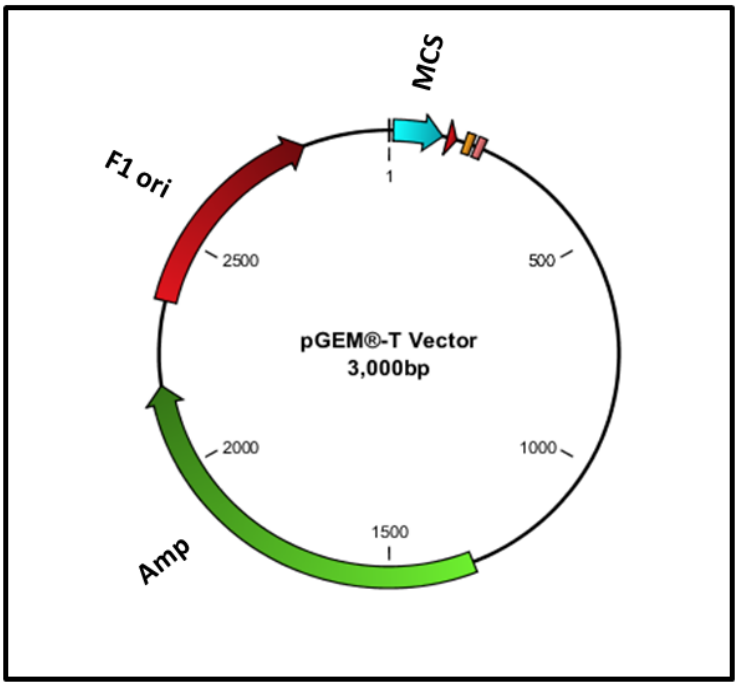


Figure 3.2 Map of **pGEMT** Vector, Promega, Catalog number A3600

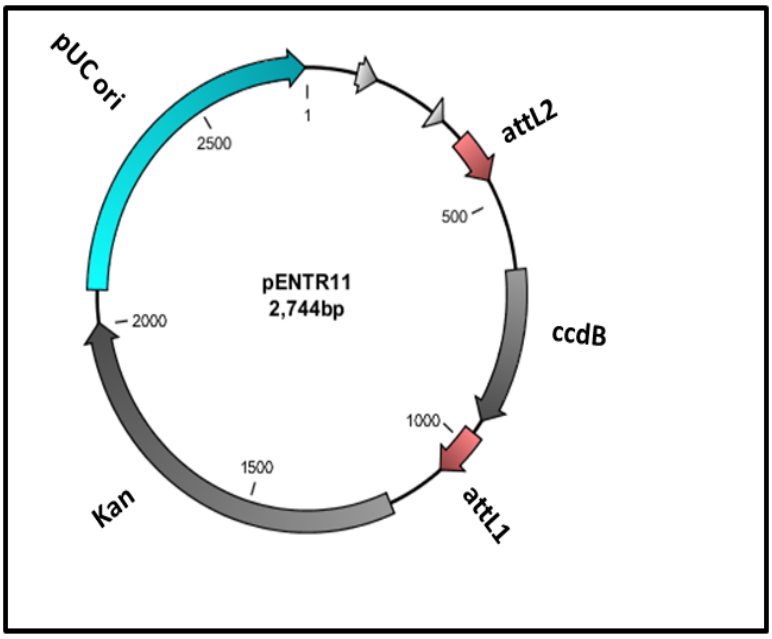


Figure 3.3 Map of **pENTR 11** Vector, Invitrogen, Catalog number A10467

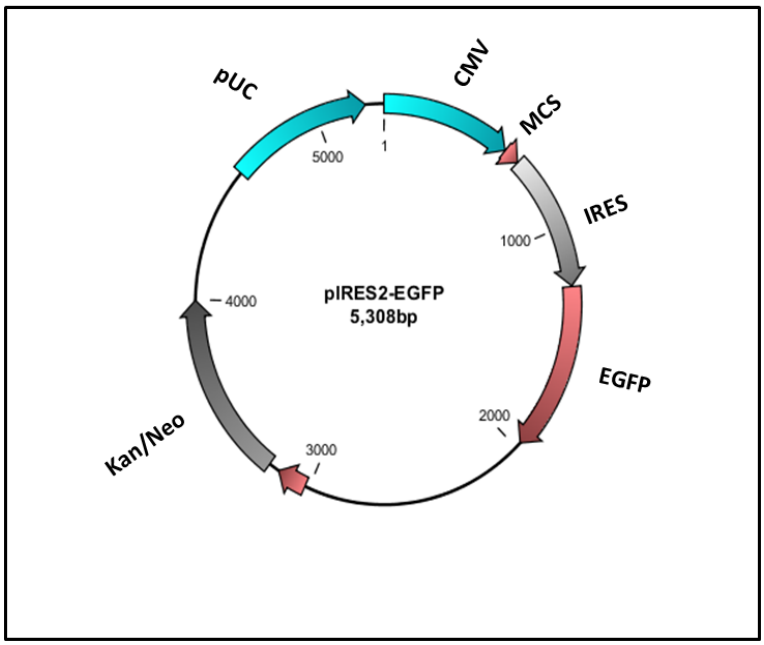


Figure 3.4 Map of **pIRES 2 EGFP** Vector, Clontech., Catalog number 6029-1

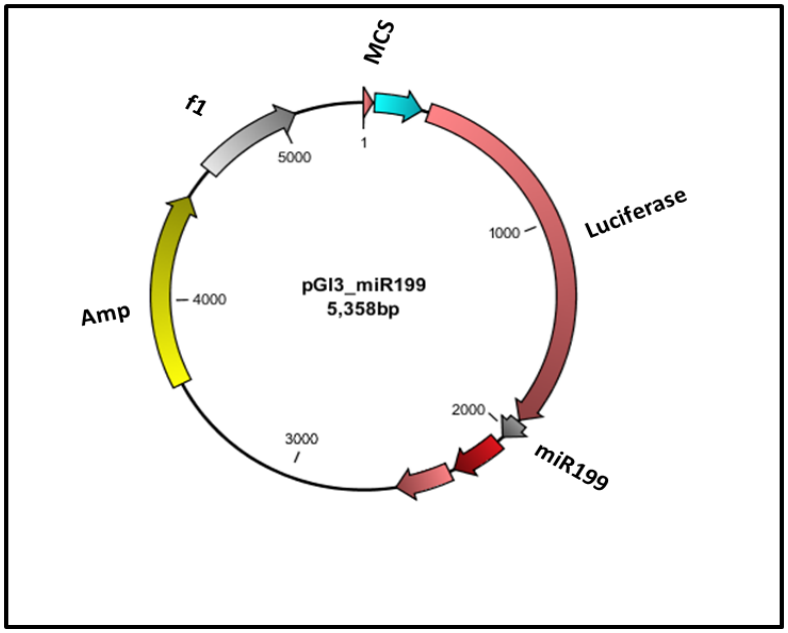


Figure 3.5 Map of **pGl3_miR-199** Vector .

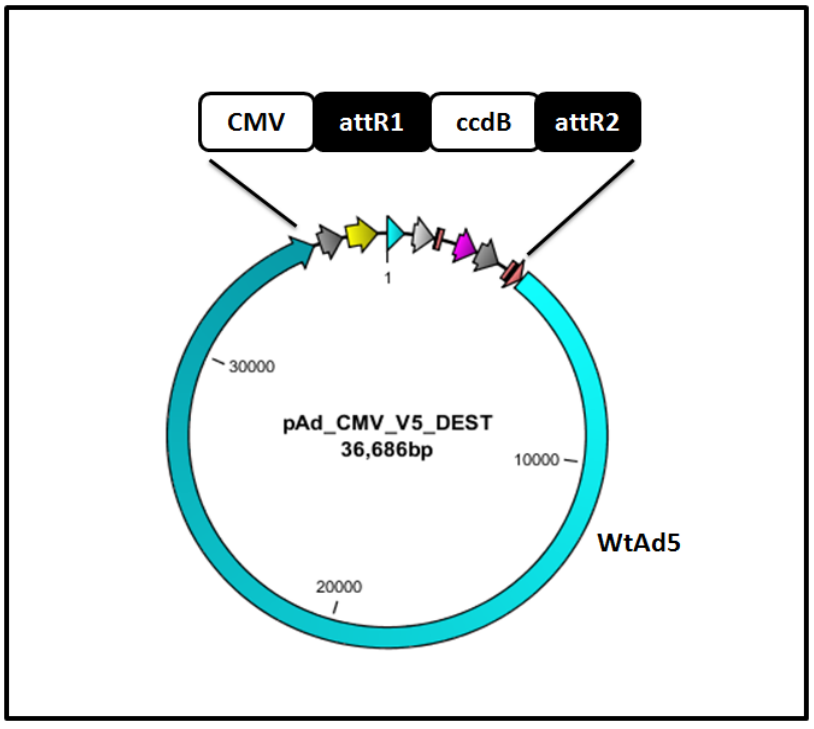


Figure 3.6 Map of **pAd/CMV/V5-Dest** Vector, Invitrogen, Catalog number V493-20

3.2 Cell lines

The hepatocellular carcinoma cell line HepG2 (ATCC HB-8065) was obtained from the America Type Culture Collection (ATCC, Manassas, VA). The human embryonal kidney cells, 293FT (R700-07), transformed with the SV40 large T antigen were obtained from Invitrogen (Carlsbad, CA). Cell lines were propagated and maintained in Dulbecco's Modified Iscove's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 0.1% Gentamycin and 1% L.Glutamine.

To produce stable clones expressing miR-199, HepG2 cells were transfected with 2µg of a miR-199 expressing plasmid, pIres-miR-199, using Lipofectamine 2000 (Invitrogen, Catalog No. 11668-027) according to manufacturer's instruction. In the same way, to produce stable clones expressing the *firefly* Luciferase reporter gene, HepG2 cells were transfected with 2 µg of a Luciferase expressing vector, pIres-Luc. After 24 hours the cell were harvested and diluted into T75 flask, then subjected to selection with 700µg/ml G418 (Roche) for 2 weeks.

3.3 Production of the recombinant Adenovirus

The Gateway® technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (155) to provide a rapid and highly efficient way to clone a gene of interest into an adenoviral vector. To generate the desired virus clone, an LR recombination reaction is performed between the entry clone and a Gateway® destination vector (Invitrogen, catalog No. V493-20). The attL x attR reaction is mediated by Gateway® LR Clonase™ II enzyme mix (Invitrogen, catalog No. 11789100), which contains the bacteriophage lambda recombination proteins Int and Xis, the *E. coli* IHF. To perform the LR recombination reaction, 300 ng of entry clone and 300 ng of destination vector are mixed with the LR Clonase™ II enzyme mix and incubated at 25^{o} C overnight. Competent *E.Coli* are transformed with the product of the recombination reaction and plasmid DNA is purified with the QIAGEN Plasmid Mini Kit (Qiagen, catalog No. 12125).

PacI linearized plasmid DNA is transfected into HEK-293T cells by Lipofectamine 2000 (Invitrogen, Catalog No. 11668-027). After 5 days the cells were harvested and subjected to three freeze-thaw cycles by freezing in 80°C and thawing in a 37 °C water bath to release the virus from the cells. For concentration of adenovirus particles, the protocol of SBI (system Biosciences, USA) using PEG-it™ Virus Precipitation Solution (SBI, catalog No. LV810A-1; LV825A-1) was used. The steps involved in virus production are summarized in **Figure 3.7**.

Titration of the virus was performed by quantitative Real-Time PCR (qRTPCR). Viral DNA was extracted using QIAamp DNA mini kit (Qiagen, catalog No. 51304) and quantified by qRT-PCR, using EVA Green (Biotium Inc, Hayward, CA, USA). All reactions were carried out in a 12.5 ul volume. Primers were as follows: wtAd5 F, 5′ CGCATACGAGCAGACGGTGAAC-3′; wtAd5-R, 5′- GCACTATAAGGAACAGCTGCGCC -3′; 18s-F, 5′-AGCAGCCGCGGTAATTCCAGCT-3′; 18s-R 5′-CGGGACACTCAGCTAAGAGCATC-3′. PCR was performed by initial denaturation at 95°C for 15min followed by 40 cycles of 30s at 95°C, 30s at 58°C and 30s at 72°C. The threshold cycle (Ct) values of each sample were used in the post-PCR data analysis. Each sample was analyzed in triplicate. For the standard curve serial dilutions of purified WtAd5 DNA was generated.

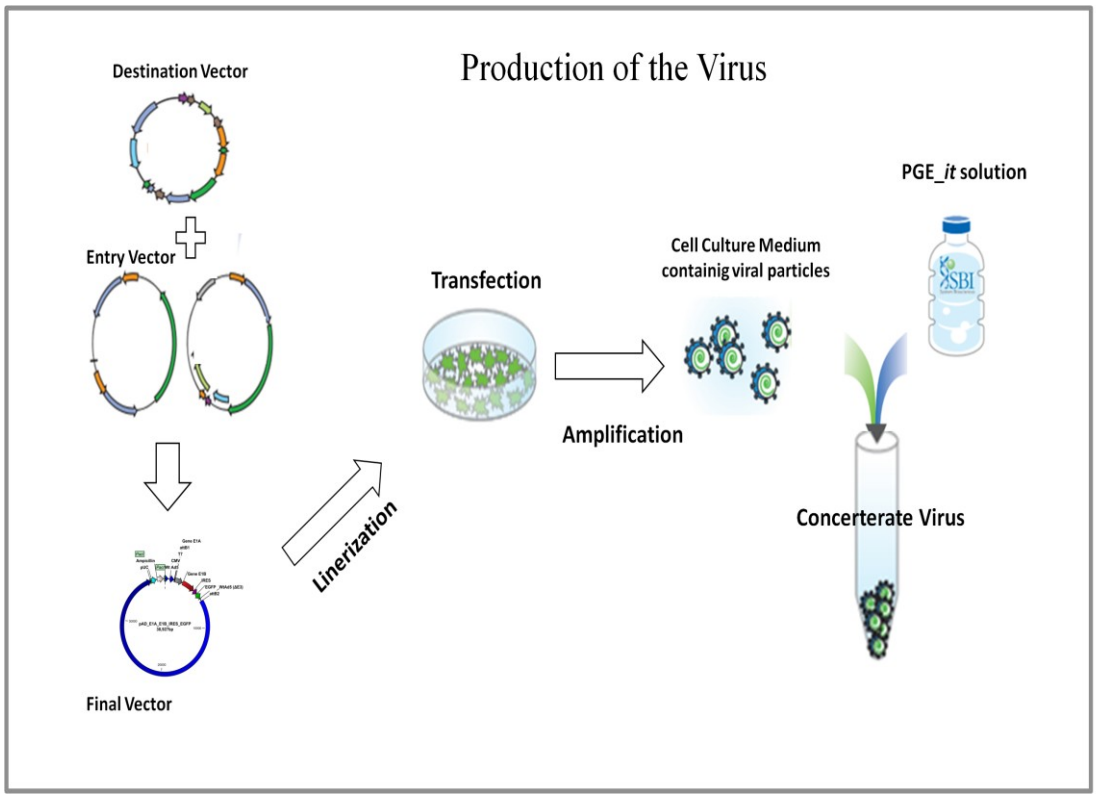


Figure 3.7 Virus production. Diagram of the steps involved in virus production: LR recombination reaction between Destination and Entry Vectors; linearization of the final vector and transfection in 293 FT cells; precipitation of the virus with PEG-it

3.4 E1A DNA analysis by Real-Time PCR

To monitor viral replication, HepG2 and HepG2_199 cells were seeded in 24-well plates at a density of 7×10^4 cells/well, cultured for 24 h, and infected with Ad-199T or with Ad-Control at multiplicity of infection (MOI) of 1×10^7 pfu/cell. The infected cells were harvested 24 h, 48 h, 72 h, 96 h and 120 h after infection and **t**otal DNA was extracted with the QiAmp DNA extraction kit (Qiagen, Catalog No. 51304) according to the manufacturer's instructions. For quantitative viral DNA detection, 50 ng of DNA was used for the PCR reaction using EVA Green (Biotium Inc, Hayward, CA, USA) . All reactions were carried out in a 12.5 ul volume. Primers were as follows: wtAd5-F, 5′-CGCATACGAGCAGACGGTGAAC-3′; wtAd5-R, 5′-GCACTATAAGGAACAGCTGCGCC -3′; 18s F, 5′-AGCAGCCGCGGTAATTCCAGCT-3′; 18s R, 5′-CGGGACACTCAGCTAAGAGCATC-3′. PCR was performed by initial denaturation at 95°C for 15 min followed by 40 cycles of 30s at 95°C, 30s at 58°C and 30s at 72°C. The threshold cycle (Ct) values of each sample were used in the post-PCR data analysis. Each sample was analyzed in triplicate. Fluorescence measurements were completed using a Biorad-Chromo4 thermal cycler real-time PCR instrument.

3.5 E1A expression analysis by Real-Time PCR

Analysis of E1A gene expression was carried out using EVA Green-based real-time PCR detection. Total RNA was extracted from frozen liver tissues after homogenization, with Trizol® reagent (Invitrogen Catalog No. 15596-026) according to the manufacturer's instructions. 200 ng of purified RNA were retro-transcribed and 5 µl of cDNA was used for the PCR reaction using EVA Green (Biotium Inc, Hayward, CA, USA). All reactions were carried out in a 12.5 ul volume. Primers were as follows: wtAd5-F, 5′-CGCATACGAGCAGACGGTGAAC-3′; wtAd5-R, 5′-GCACTATAAGGAACAGCTGCGCC -3′; 18s-F, 5′-AGCAGCCGCGGTAATTCCAGCT-3′; 18s-R, 5′-CGGGACACTCAGCTAAGAGCATC-3′. PCR was performed by initial denaturation at 95°C for 15 min followed by 40 cycles of 30s at 95°C, 30s at 58°C and 30s at 72°C. The threshold cycle (Ct) values of each sample were used in the post-PCR data analysis. Each sample was analyzed in triplicate. E1A expression levels were normalized against 18s housekeeping gene. Fluorescence measurements were completed using a Biorad-Chromo4 thermal cycler real-time PCR instrument.

3.6 E1A protein analysis.

To evaluate the expression of adenoviral E1A protein, HepG2 and HepG2-199 cells were seeded in 24-well plates at a density of 7×10^4 cells/well, cultured for 24 h, and infected with Ad-199T or with Ad-Control at multiplicity of infection (MOI) of 1×10^7 pfu/cell. Two days later, cells were harvested and lysed by using RIPA lysis buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) with complete protease inhibitor cocktails (Sigma, St Louis, MO). Homogenates were then centrifuged at 13000 rpm for fifteen minutes at 4°C and supernatants were collected and analyzed by Western blot to assess E1A protein expression with a polyclonal anti-E1A antibody (Santa Cruz Biotechnology, Catalog No. sc-430) and a rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Catalog No. sc-2749) Signal was revealed with the LiteAblot Turbo Extra-Sensitive Chemiluminescent Substrate (Euroclone, catalog No. EMPO 12001). Digital images of autoradiographies were acquired with Fluor-S MultiImager and band signals were acquired in the linear range of the scanner using a specific densitometric software (Quantity One). After autoradiography acquisition, the membranes were reprobed for 1 h at room temperature with anti-β-tubulin polyclonal antibody (Santa Cruz Biotechnology, Catalog No. H-235, sc-9104) diluted 1:1000.

3.7 *In vivo* experiments

Animal protocol was approved by institutional ethical committee. The mice were mantained in a vented cabinet at 25°C with 12-hour light-dark cycle and provided food and water ad libitum. The mouse strain B6D2F2 is an intercross between B6D2F1 (C57Bl6/J x DBA/2J) animals. Three-days newborn mice received one intra-hepatical injection of 2×10^6 HepLuc cells re-suspended in 40 ul of PBS. The day after the animals were injected intra-hepatically with viruses. All mice were subjected to autopsy and livers were partly fixed in 10% formalin and partly frozen in liquid nitrogen. Tissues were collected at 24, 48 and 72 hours after the first viral injection. Total liver DNA was extracted using the QIAmp DNA Mini Kit, following the manufacturer's instructions (Qiagen, Catalog No. 51304).

3.8 *In Vivo* Imaging System (IVIS).

In vivo bioluminescent imaging was performed with a ultra low noise, high sensitivity cooled CCD camera, mounted on a light tight imaging chamber (IVIS 100 System™, Xenogen, Roissy, France).

Tracking, monitoring and quantification of signals were controlled by the acquisition and analysis software Living ImageH (Xenogen Corp, Alameda, CA). D-luciferin was injected intraperitoneally at a dose of 150 mg/kg body weight (30 mg/ml luciferin) to anesthetized (1–3% isoflurane) animals 15 minutes before image acquisition. Anesthetized mice were then placed in the IVIS™ Imaging System and imaged. Three-four mice were imaged at each time. Regions of interest from displayed images were identified around the tumor sites and were quantified as total photon counts or photons/s using the Living ImageH software (Xenogen Corp., Alameda, CA).

3.9 Tables

The **Table 3.1** provides a list of the primers used in cloning steps, sequencing reactions and Real-Time PCR.

Name	**Sequence**	**Annealing Temp**
E1A-E1B-EcoRI- pShuttle-539-F	GAATTCCGCGCACCATGGTGTCGACG	64.0 ºC
E1A-E1B-EcoRI-pShuttle-3569-R	GAATTCGCCACGCCCACACATTTCAGTACCTC	63.5 ºC
E1A-E1B -1465-R	GATACATTCCACAGCCTGGC	56.0 ºC
E1A-E1B-1325-F	CCCGACATCACCTGTGTCTA	56.2 ºC
E1A-E1B-2358-R	GCTAGATTCCTAGCCTCCTCTG	56.2 ºC
E1A-E1B-2233-F	ACCCTCGGGAATGAATGTTG	55.1 ºC
E1A-E1B-3226-R	GCATTGGTAAGGTAGGAACACC	55.7 ºC
E1A-E1B-3086-F	TCTCAGATGCTGACCTGCTC	56.5 ºC
pGl3-MluI -1851 F	ACGCGT AGG TCT TAC CGG AAA ACT CG	54.4 ºC
pGl3-MluI -2220-R	ACGCGT CCC CCT GAA CCT GAA ACA TA	54.3 ºC
IRESEGFP-1061-F	TGGCTCTCCTCAAGCGTATT	56.0 ºC
IRESEGFP-1863-R	TGCTCAGGTAGTGGTTGTCG	56.9 ºC
pENTR11-F	CCAACTTTGTACAAAAAAGC	51.0 ºC
EGFP-1584-R	AAGTCGTGCTGCTTCATGTG	55.9 ºC
18s-616-F	AGCAGCCGCGGTAATTCCAGCT	58.0 ºC
18s-784-R	CGGGACACTCAGCTAAGAGCATC	58.0 ºC
T7	TAATACGACTCACTATAGGG	56.0 ºC
SP6	GATTTAGGTGACACTATAGA	54.0 ºC
wtAd5-11523-F	CGCATACGAGCAGACGGTGAAC	60.3 ºC
wtAd5-11681-R	GCACTATAAGGAACAGCTGCGCC	60.4 ºC
β actina-RNA-1249-F	CATTGCCGACAGGATGCA	58.0 ºC
β actina-RNA-1399-R	GCTGATCCACATCTGCTGGA	58.0ºC

3.10 Statistical analysis.

Statistical significance was determined using a double tailed Student's t test. For all data analyzed, a significance threshold of $P < 0.05$ was assumed. In all figures, values were expressed as mean ± standard deviation (SD).

4. RESULTS

4.1 Design and construction of Adenoviral vectors

First aim of this study was the production of novel recombinant adenovirus as an oncolytic agent for the treatment of liver cancer. This adenovirus vector was designed to be replication-competent. Hence, it expresses E1A/E1B genes, which are required for adenovirus replication. To make replication miR-199-dependent, the E1A gene was designed to be engineered through the introduction of miR-199 target sequences at its 3'end. The overall vector construction strategy is schematically depicted in **Figure 4.1.** The various steps are detailed in the paragraphs that follow. A control adenovirus, pAd-Control, which lacks miR-199 target sequences, was also developed.

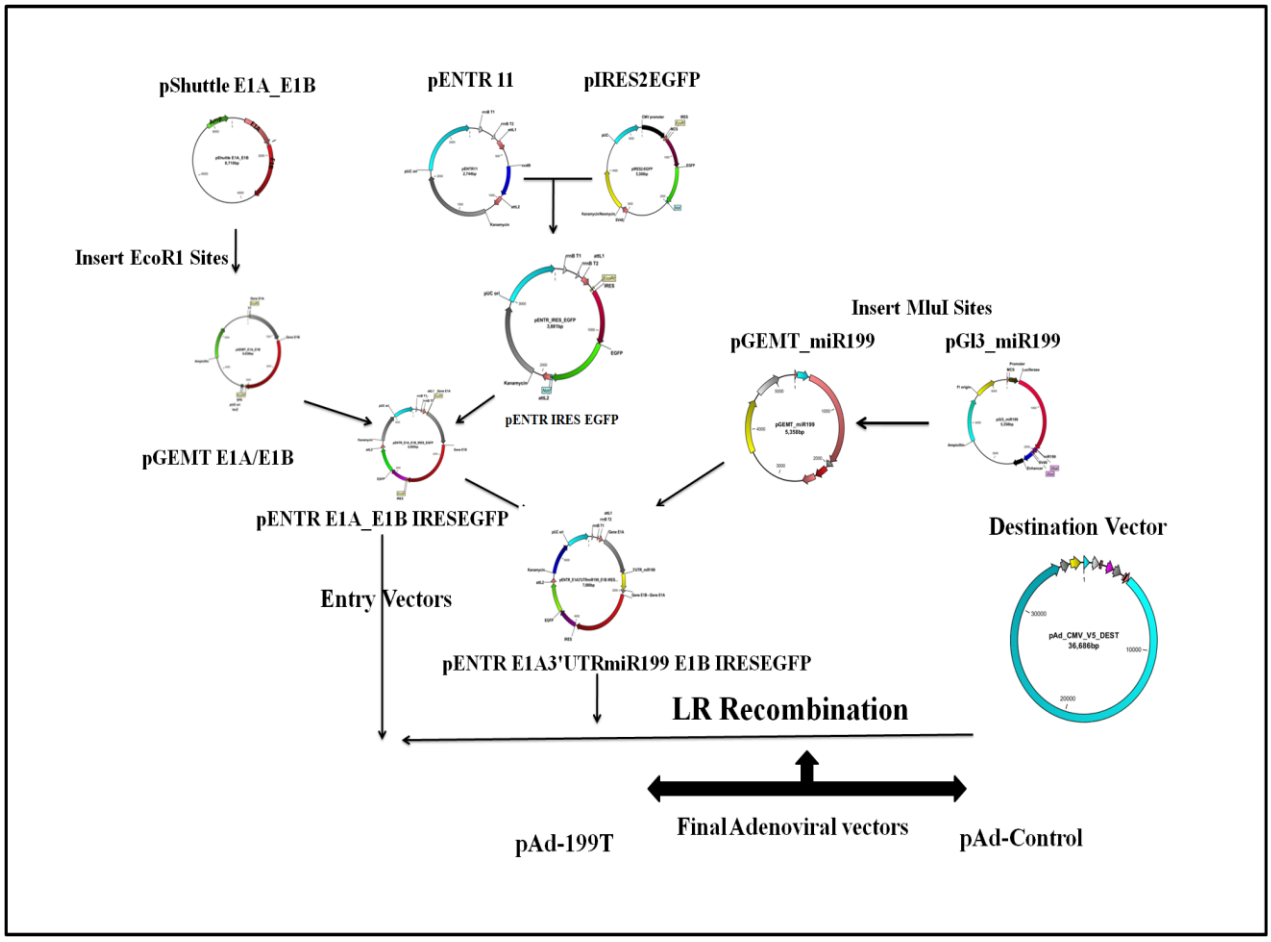


Figure 4.1 Vectors construction strategy. Diagram showing the cloning steps involved in pAD-199T and pAd-Control construction.

4.1.1 Construction of the "starting vector" pGEM_E1A/E1B

pGEMT-E1A/E1B (**Figure 4.2 A**) was obtained by cloning the E1A/E1B coding sequences into pGEM-T Easy vector. E1A/E1B coding sequences were amplified by PCR from the pShuttle E1A/E1B vector (**Figure 3.1**), using the primers E1A-E1B-EcoRI-pShuttle-539-F/ E1A-E1B-EcoRI-pShuttle-3569-R (**Figure 4.2 B**). The primers contain the sites for the restriction enzyme EcoR I. The PCR product was ligated into the pGEM-T Vector. (**Figure 3.2**) and transformed *E.Coli* cells were analyzed by PCR using E1A-E1B-2233-F/ E1A-E1B-3226-R primers. The PCR analysis revealed several positive colonies (**Figure 4.2 C**). To confirm that the E1A/E1B were inserted in the vector, pGEMT-E1A/E1B was digested with EcoRI and ScaI. Three different fragments of 3030 bp, 1875 bp and 1125 bp were correctly generated (**Figure 4.2 D**), as predicted from the map.

The pGEMT_E1A/E1B vector was then sequenced to verify that no errors were inserted into the E1A/E1B coding sequences during PCR amplification from the pShuttle E1A/E1B vector. Four subsequent sequencing reaction were performed using eight different primers, listed in **Table 3.1** . Chromatogram data analysis shows perfect matching between the generated and expected sequence, confirming that no mutations have occurred inside the cloned E1A/E1B genes.

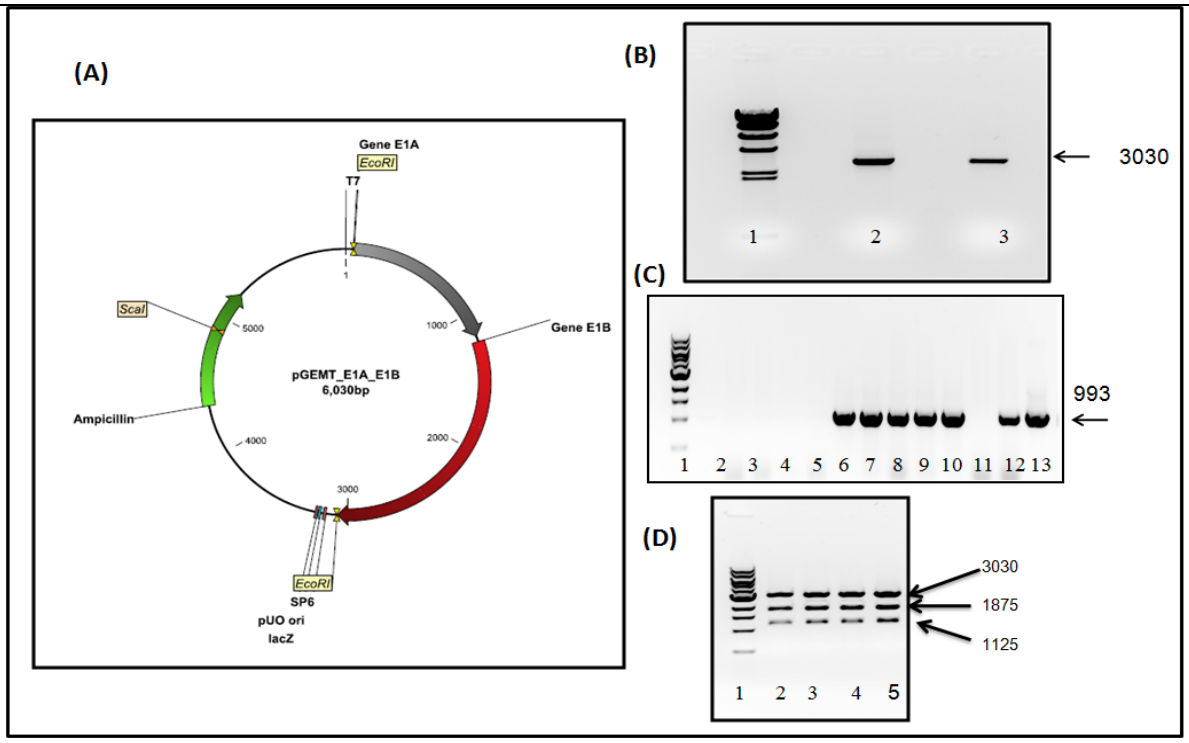


Figure 4.2. Construction of the "starting vector" pGEM_E1A/E1B A. map of the vector pGEMT_E1A/E1B (6030 bp): E1A and E1B genes, the ampicillin resistance gene and the replication origin in *E.Coli* are shown. **B**. Electrophoresis of PCR product generated by E1A-E1B-EcoRI-pShuttle-539-F/ E1A-E1B-EcoRI-pShuttle-3569-R primers from the pShuttle E1A/E1B vector; 1: Lambda HindIII molecular weight marker; 2, 3: 3030 bp fragment corresponding to the E1A/E1B coding sequences. **C**. Electrophoresis of PCR products generated by E1A-E1B-2233-F/ E1A-E1B-3226-R primers from eleven isolated colonies; 1: 1kb molecular weight marker; 2-5 and 11: negative colonies, 6-10 and 12: positive colonies, 13: positive control; the expected product size was 993 bp. **D**. Electrophoresis of pGEMT_E1A/E1B digested with EcoRI and ScaI (2-5); 1: 1kb molecular weight marker. Three different fragments of 1125 bp, 1875 bp and 3030 bp were correctly generated.

4.1.2 Construction of the "intermediate vector" pENTR_IRES/EGFP

To construct the final entry vector, an intermediate vector was generated. To this aim, the vector pIRES2EGFP (**Figure 3.4**) was digested with the restriction enzymes EcoRI and NotI, generating two fragments: one of 3961 bp, corresponding to the vector backbone; the other of 1347 bp , corresponding to the IRES (Internal Ribosome Entry Site) and the EGFP (Green Fluorescent Protein Enhancer) coding sequence. This fragment was then cloned into pENTR 11 (**Figure 3.3**) generating the pENTR_IRES/EGFP vector (**Figure 4.3 A**). Competent *E.coli*, transformed with the new construct, were analyzed by PCR with primers IRES_EGFP-1061-F /IRES_EGFP-1863-R. The PCR analysis revealed several positive colonies (**Figure 4.3 B**). To confirm that the IRES EGFP sequences were inserted in the vector, pENTR_IRES/EGFP was digested with EcoRI and

NotI. Two fragment of 2314 bp and 1347 bp were correctly generated (**Figure 4.3 C**), as predicted from the map

Figure 4.3 Construction of the pENTR_IRES/EGFP "intermediate" vector. A. map of the vector pENTR_IRES/EGFP (3661 bp): the internal ribosome entry site (IRES), the Enhanced Green Fluorescent Protein (EGFP), attL1 and attL2 sites are indicated. **B**. Electrophoresis of PCR product using IRES_EGFP-1061-F/ IRES_EGFP-1863-R primers; 1: 1kb molecular weight marker ; 2-9: positive colonies, product size of 802 bp. **C.** Electrophoresis of restriction digestion of pENTR_IRES/EGFP with EcoRI and NotI (2-4): 1 lambda HindIII molecular weight marker. Two different fragments of 2314 bp and 1347 bp are correctly generated.

4.1.3 Construction of the "Control entry vector" pENTR_E1A/E1B IRES EGFP

To generate the pENTR_E1A/E1B IRES EGFP "control entry vector" (**Figure 4.4 A**). pGEMT/E1A_E1B was digested with EcoRI and NotI, and the E1A/E1B 3030 bp fragment was cloned in the pENTR_IRES EGFP vector. Transformed *E.coli* were analyzed by PCR with primers E1A-E1B-2233-F/ E1A-E1B-3226-R. The PCR analysis revealed several positive colonies (**Figure 4.4 B**). To confirm that the E1A/E1B coding sequences were inserted in the vector, pENTR_E1A/E1B IRES EGFP was digested with EcoRI.(generating two fragments of 3030 bp, and 3655 bp) (**Figure 4.4. C**), and with HindIII (generating two fragments of 5656 bp, and 1029 bp) (**Figure 4.4 D**), as predicted from the map.

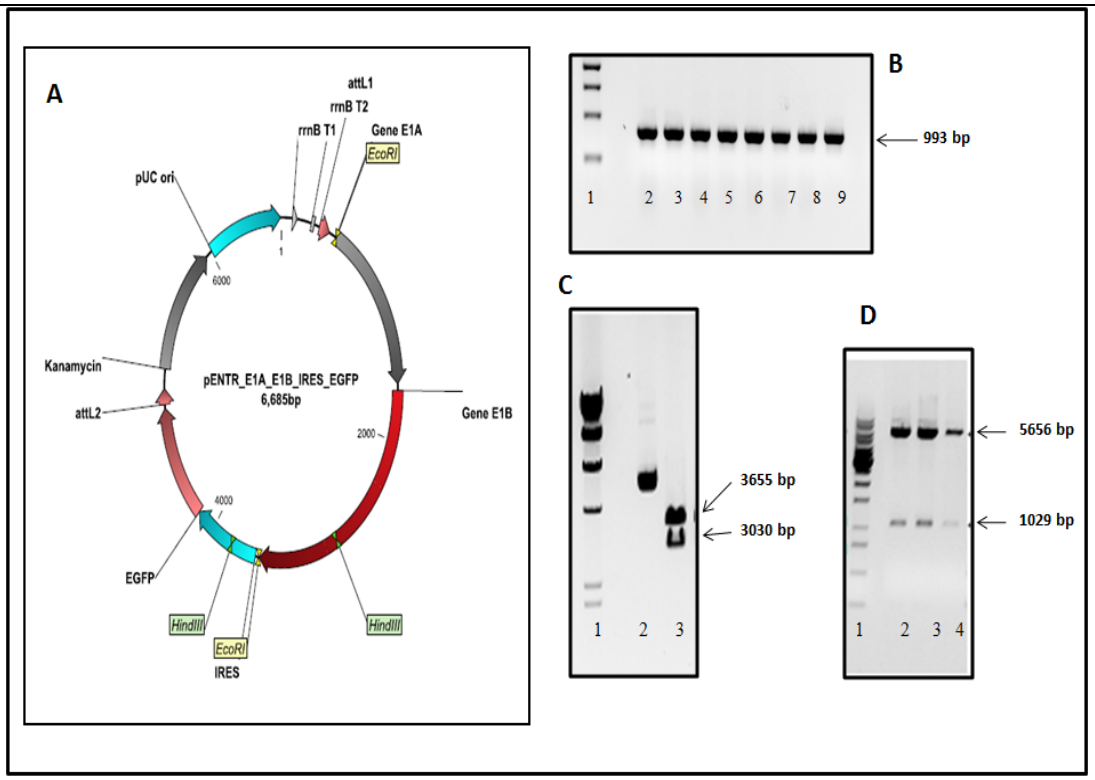


Figure 4.4 Construction of pENTR_ E1A/E1B_IRES EGFP. **A.** Map of the vector (6685 bp): IRES, EGFP and the E1A/E1B coding sequence are indicated. **B.** Electrophoresis of PCR product using E1A-E1B-2233-F/ E1A-E1B-3226-R, primers; 1: 1kb molecular weight marker ; 2-9: positive colonies, product size 993 bp , **C.** Electrophoresis of pENTR_E1A/E1B_IRES EGFP undigested (2) and digested with EcoRI (3), 1: lambda HindIII molecular weight marker; Two different fragments of 3030 bp, and 3655 bp were generated. **D.** Electrophoresis of pENTR_E1A/E1B_IRES_EGFP digested with HindIII(2-4); 1: Gene Ruler 1kb molecular weight marker;. Two different fragments of 5656 bp, and 1029 bp were correctly generated.

4.1.4 Construction of the "Entry vector" pENTR_E1A T199 /E1B IRES EGFP

To generate the "entry vector" pENTR_E1A T199 /E1B IRES EGFP, many intermediate steps are necessary, involving different constructs. The vector pGL3 T199 (**Figure 3.5**) was used as donor of miR-199 responsive sequences. This vector contains four tandem repeats of a sequence complementary to the mature hsa-miR-199a_3p sequences (http://microrna.sanger.ac.uk/), flanked by XbaI sites. The target sequence responsive to miR-199 (named "T199"), was amplified from pGL3 miR-199, using the pGl3-Mlu1-1851-F/ pGl3-Mlu1-2220-R primers, containing the sites for the restriction enzyme Mlu1. The 395 bp fragment, corresponding to T199, was cloned in the pGEM_T, vector, generating pGEMT T199. pGEMT T199 was then digested with the restriction enzyme MluI; the T199 fragment, was cloned into the pENTR_E1A/E1B IRES EGFP vector, generating the pENTR_E1A T199 /E1B IRES EGFP "entry vector" (**Figure 4.5 A**). Competent

E.coli were transformed with the construct and colonies were analyzed by PCR using the pGl3-MluI-1843-F/ pGl3-MluI-2238-R primers. (**Table 3.1**) The PCR analysis revealed several positive colonies (**Figure 4.5 B**). To further verify the correct cloning of the T199 fragment in the pENTR_E1A T199 /E1B IRES EGFP, the vector was digested with the restriction enzyme MluI ; two different fragments of 395 bp and 6685 bp, corresponding respectively to T199 and to the vector backbone, were correctly generated. (**Figure 4.5 C**).

To verify that T199 was correctly "in sense" inserted respect to the E1A coding region, the T199 sequence was amplified from pENTR_E1A T199/E1B IRES EGFP using E1A-E1B-1325-F primer (complementary to the E1A/E1B sequences) and with pGl3-Mlu-12238-R (complementary to the vector sequence) (**Table 3.1**). The PCR originated a 624 bp fragment corresponding to the "in sense" T199 sequence. Furthermore, to verify the integrity and orientation of the cloned sequence, the vector pENTR_E1A 3'UTR miR-199 /E1B IRES EGFP was sequenced using the primer E1A-E1B-1325-F (**Table 3.1**). The cloned fragment was found to be intact and correctly oriented.

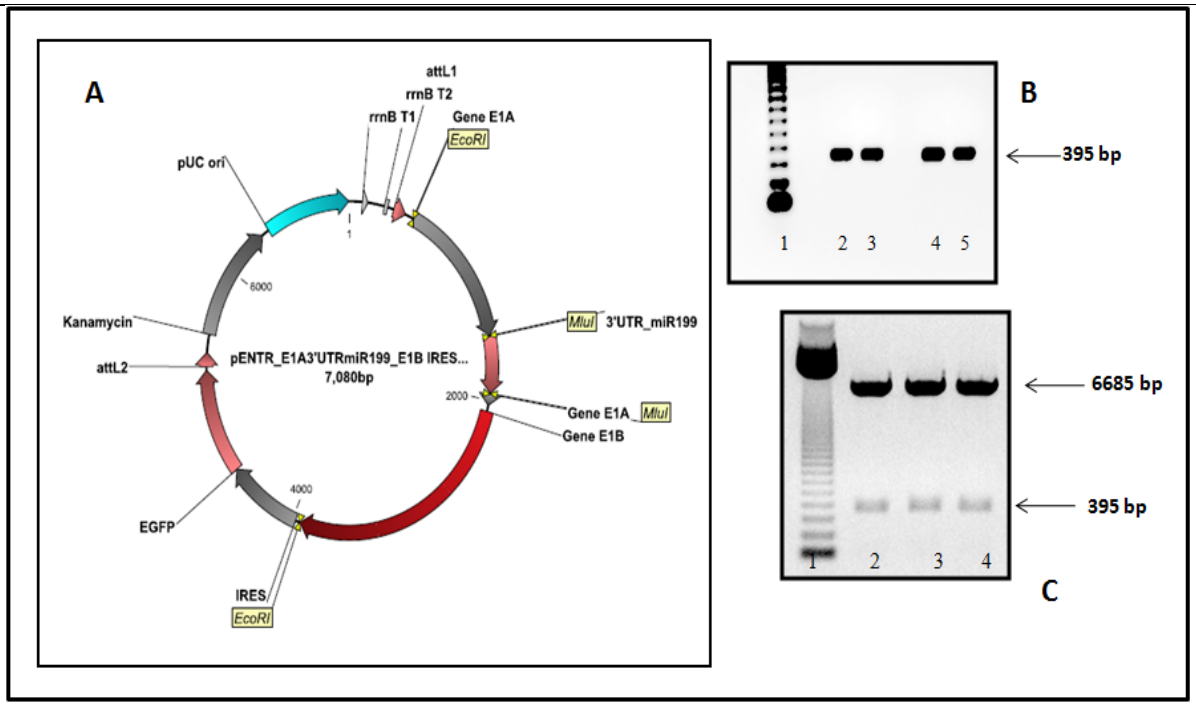
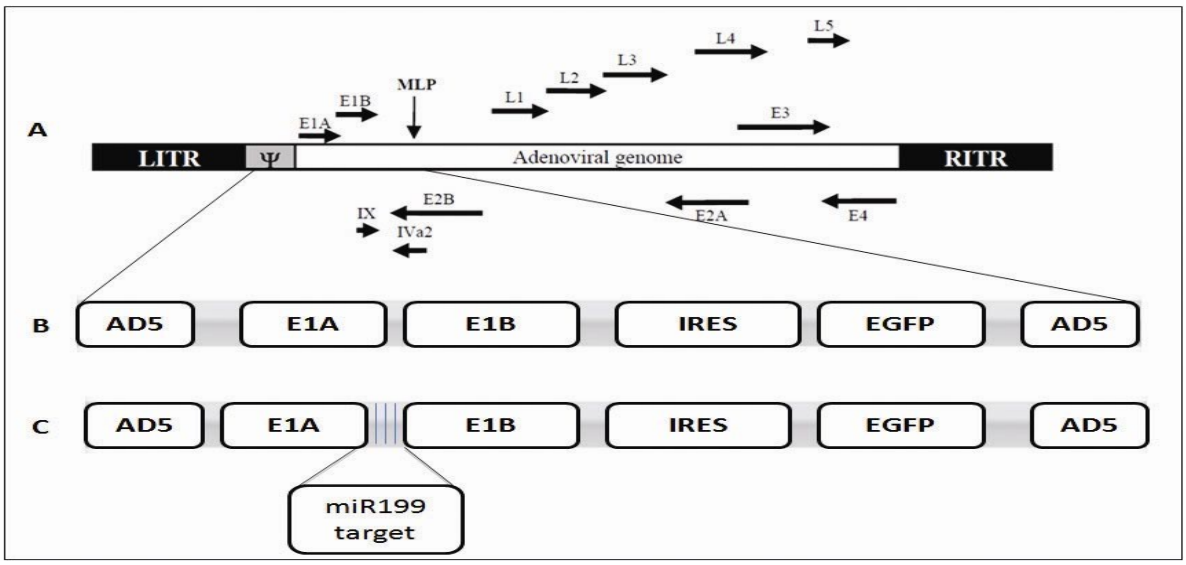


Figure 4.5 Construction of pENTR_E1A T199 /E1B IRES EGFP. **A.** Map of the vector (7080 bp): IRES, EGFP, E1A/E1B coding sequence and the target sequence responsive to miR-199 (T199) are indicated. **B**. Electrophoresis of PCR product using pGl3-Mlu1-1851-F/ pGl3-Mlu1-2220-R primers; 1: 123 bp molecular weight marker; 2-5: positive colonies, product size 395 bp. **C**. Electrophoresis of restriction digestion of pENTR_E1A T199/E1B IRES EGFP with MluII (2-4); 1: 123 bp molecular weight marker.Two different fragments of 6685 bp and 395 bp were correctly generated.

4.1.5 Construction of final Adenoviral vectors

The adenovirus used in our study was derived from the viral vector pAd/CMV/V5-Dest (pAd Dest), based on the genome of adenovirus type 5, and lacking E1-E3 genes necessary for viral replication (**Figure 4.6**). The E1A T199/E1B cassette was inserted in the pAd Dest and the recombinant adenovirus was produced, generating a conditionally replicative adenovirus (Ad-199T) expressing the E1A gene under miR-199 control. A control adenovirus (Ad-Control) lacking the T199 sequence, was also generated.



hsa-miR-199a-3p (MIMAT0000232)	ACAGUAGUCUGCACAUUGGUUA
Target of miR-199	5'TAACCAATGTGCAGACTACTGTccTAACCAATGTGCAGACTACTGTccTAACCAATGTGCAGACTACTGTccTAACCAATGTGCAGACTACTGTcc-3'

Figure 4.6 Schematic representation of the Adenovirus type 5 genome and of the recombinant adenoviruses generated. **A**. adenovirus type 5 genome: the viral early and late genes and the Inverted Terminal Repeat (ITR) are indicated. **B.** Ad-Control genome: the viral E1A and E1B genes, IRES and EGFP are indicated. **C.** Ad-199T genome: the target sequence responsive to miR-199 (T199) cloned at the E1A 3' UTR is indicated. The sequences of miR-199 and of T199 are also shown.

4.1.5.1. Construction of pAd-Control vector

The adenoviral vector pAd-Control was obtained by LR Recombination Reactions between the Control Entry Vector pENTR_E1A/E1B IRES EGFP and the adenoviral Destination Vector pAd/CMV/V5-Dest (**Figure 4.7 A and C**). The *in vitro* recombination between the two construct occurs between the attR1 and attR2 sites of the Destination vector and the attL1 and attL2 sites of the Entry vector. The resulting recombination reaction product was then used to transform *E. coli* cells, and the transformed bacteria colonies were analyzed by PCR using primers E1A-E1B-2233-F/ E1A-E1B-3226-R, complementary to the E1A/E1B sequences. The PCR analysis revealed several positive colonies (**Figure 4.7 B**), with an expected band size of 993 bp.

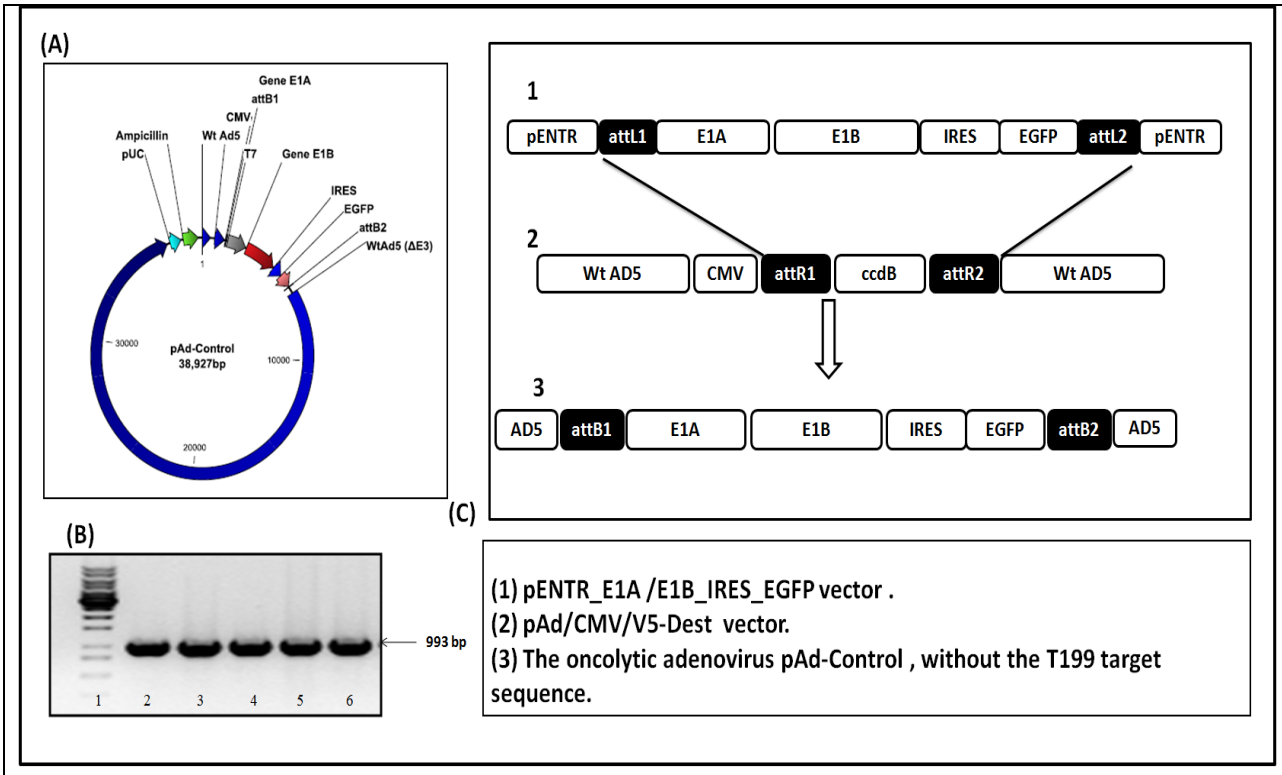


Figure 4.7 Construction of pAd-Control. A. map of the vector: the type 5 Adenovirus genome, lacking the E1-E3 genes, the E1A/E1B and EGFP coding sequences under the CMV promoter, are indicated. **B**. Electrophoresis of PCR products using E1A-E1B-2233-F/ E1A-E1B-3226-R primers on colonies obtained following recombination between Entry and Destination vectors: 1. Gene Ruler 1Kb molecular weight marker ; 2-6 :positive colonies, product size 993 bp. **C**. Diagram showing the site specific recombination between pENTR_E1A/E1B IRES EGFP (1) and pAd/CMV/V5-Dest (2), generating pAd-Control (3).

4.1.5.2. Construction of pAd-199T vector

The adenoviral pAd-199T vector was obtained by recombination between the Entry Vector pENTR_E1A /T199 /E1B_IRES_EGFP and the adenoviral Destination Vector pAd/CMV/V5-Dest (**Figure 4.8 A and C**). The *in vitro* recombination between the two construct occurs between the attR1 and attR2 sites of the Destination vector and the attL1 and attL2 sites of the Entry vector. The resulting recombination reaction product was then used to transform *E. coli* cells, and the transformed bacteria colonies were analyzed by PCR using primers E1A-E1B-2233-F/ E1A E1B-3226-R, complementary to the E1A/E1B sequences. The PCR analysis revealed several positive colonies (**Figure 4.8 B**).

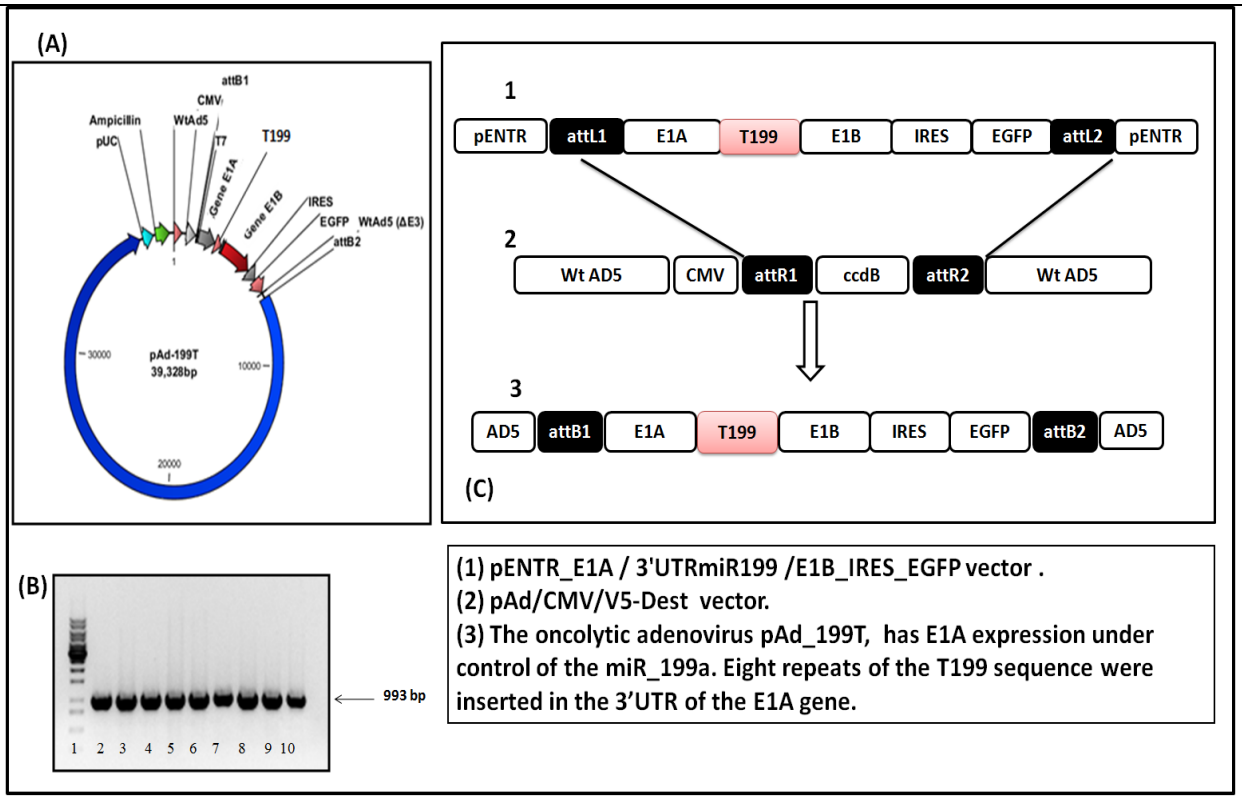


Figure 4.8 Construction of pAd-199T. A. map of the vector: the type 5 Adenovirus genome, lacking the E1-E3 genes, the E1A_T199, E1B and EGFP coding sequences under the CMV promoter, are indicated; **B**. Electrophoresis of PCR products using E1A-E1B-2233-F/ E1A-E1B-3226-R primers on colonies obtained following recombination between Entry and Destination vectors: 1. Gene Ruler 1Kb molecular weight marker ; 2-10 :positive colonies, product size 993 bp. **C**. Diagram showing the site specific recombination between pAD_E1A_3'UTR miR-199_E1B_IRES_EGFP (1) and pAd/CMV/V5-Dest (2), generating pAd-199T(3).

To verify that the new adenoviral vectors were correctly generated by the recombination reactions between the entry and the destination vectors, pAd DEST , pAd-Control and pAd-199T were

digested with the restriction enzyme HindIII. The resulting restriction patterns corresponded to those predicted on the basis of sequence information, confirming that the recombination reactions had occurred as expected (**Figure 4.9**)

A

B

pAd-199T	pAd-Control	pAd DEST
Length (bp)	Length (bp)	Length (bp)
8010	8010	8010
5324	5324	6326
4597	4597	5324
4244	4244	4597
3787	3787	3787
3731	3549	3549
3549	3330	2937
2937	2937	2081
2081	2081	75
993	993	
75	75	

Figure 4.9 Characterization of pAd-199T and pAd-Control. **A**. Electrophoresis of pAd-199T (2), pAd-Control (3) and pAd/CMV/V5-Dest (pAd DEST) (4)l after digestion with restriction enzyme HindIII. 1: Gene Ruler 1Kb molecular weight marker; 5. Lambda HindIII molecular weight marker. **B**. Size of the restriction fragments generated by HindIII digestion of the three adenoviral vectors, as predicted by the soft ware program NEBcutter. The results shows that the recombination reactions had correctly occurred.

4.1.6. Production of Adenoviruses

The viral particles of recombinant Adenoviruses were produced using the HEK 293 FT cells, derived from human embryonic kidney fibroblast. This cell line express Adenovirus E1A/E1B genes as well as the SV40 T antigen, which allows the production of viral particles at high titers. The adenoviral vectors pAd-199T and pAd-Control were first linearized by digestion with the restriction enzyme PacI to expose the ITR (Inverted Terminal Repeat) region, which is necessary for virus replication. The linearized adenoviral vectors were then transfected into HEK 293 FT cells; the virus was collected and used to infect again 293 FT cells, to obtain the final viral stock. Purified. viral particles (vp) were quantified by EVA Green-based quantitative real-time PCR using

wtAd5-11523-F/ wtAd5-11681-R primers . The titer of the produced adenoviruses were $6.24x10^{10}$ vp/ml for Ad-199T and $1.95x10^{10}$ vp/ml for Ad-Control .

Since 293 FT cells constitutively express E1A E1B genes, allowing viral replication also in replication defective recombinant adenoviruses, the replication ability of the Ad-199T and Ad-Control viruses was checked in HepG2 cells., Virus production was confirmed by the appearance of a cytopathic effect (CPE) in HepG2 cells 24 h after infection **Figure 4.10** .

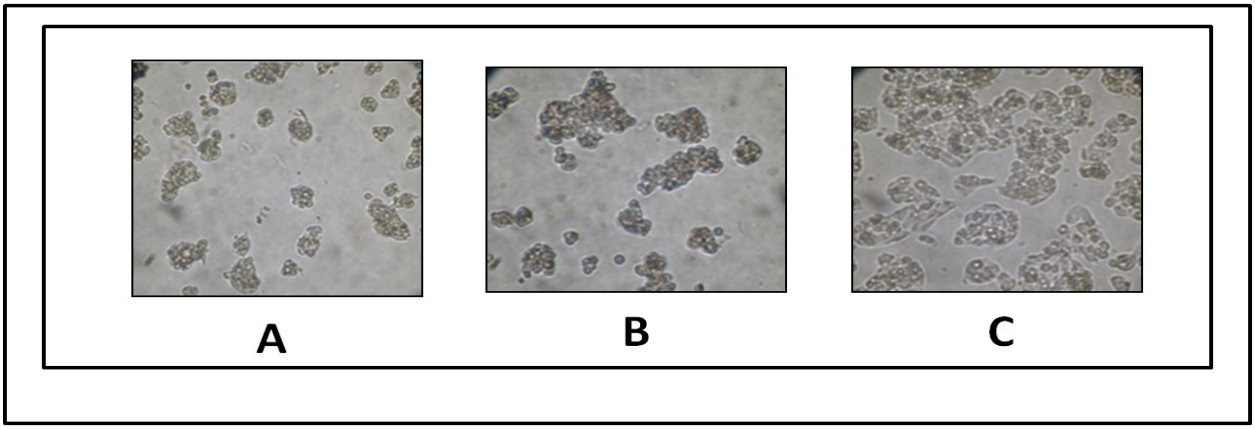


Figure No. 4.10 Ad-199T and Ad-Control replicate in HepG2 cells. HepG2 cells were infected with Ad-199T (**A**) with Ad-Control (**B**) or not infected (**C**). Virus production was confirmed by the appearance of a cytopathic effect (CPE) in HepG2 cells, observed with a light microscope (magnification 40X) 24 h after infection.

4.2 Viral replication of Ad-199T is microRNA-dependent *in vitro* and *in vivo*

The aim of this study is the production of a conditionally replicative adenovirus (CRAD) under miR-199 control. To verify if miR-199 could regulate E1A expression and viral replication, Ad-199T and Ad-Control were used to infect two different cell lines: (1) HepG2, derived from human liver carcinoma and not expressing miR-199; (2) HepG2-199, which derives from HepG2, but was engineered to constitutively express miR-199a.

To assess viral replication in absence of miR-199, HepG2 cells were infected with Ad-199T or with Ad-Control. The infected cells were harvested 24 h, 48 h, 72 h, 96 h and 120 h after infection to assess viral replication. For this purpose, DNAs extracted from infected cells were analyzed by quantitative PCR using primers specific for E1A. The result showed a progressive accumulation of

viral DNA in the cells infected with the control adenovirus and in those infected with Ad-199T. This result demonstrates that active viral replication is occurring for both viruses in this miR-199 negative cell line. (**Figure 4.11**)

To assess viral replication in the presence of miR-199, HepG2-199 cells were infected with Ad-199T or with Ad-Control. The infected cells were harvested 24 h, 48 h, 72 h,96 h and 120 h after infection to assess viral replication. DNAs extracted from infected cells were analyzed by quantitative PCR using wtAd5-11523-F/ wtAd5-11681-R primers which allows the amplification of E1A. The result showed a progressive accumulation of viral DNA in the cells infected with adenovirus control (Ad-Control), indicating that active viral replication is occurring within the cells. Conversely, in cells infected with Ad-199T the viral DNA did not increase over time, confirming that the virus replication was inhibited by the presence of miR-199 (**Figure 4.11)**.

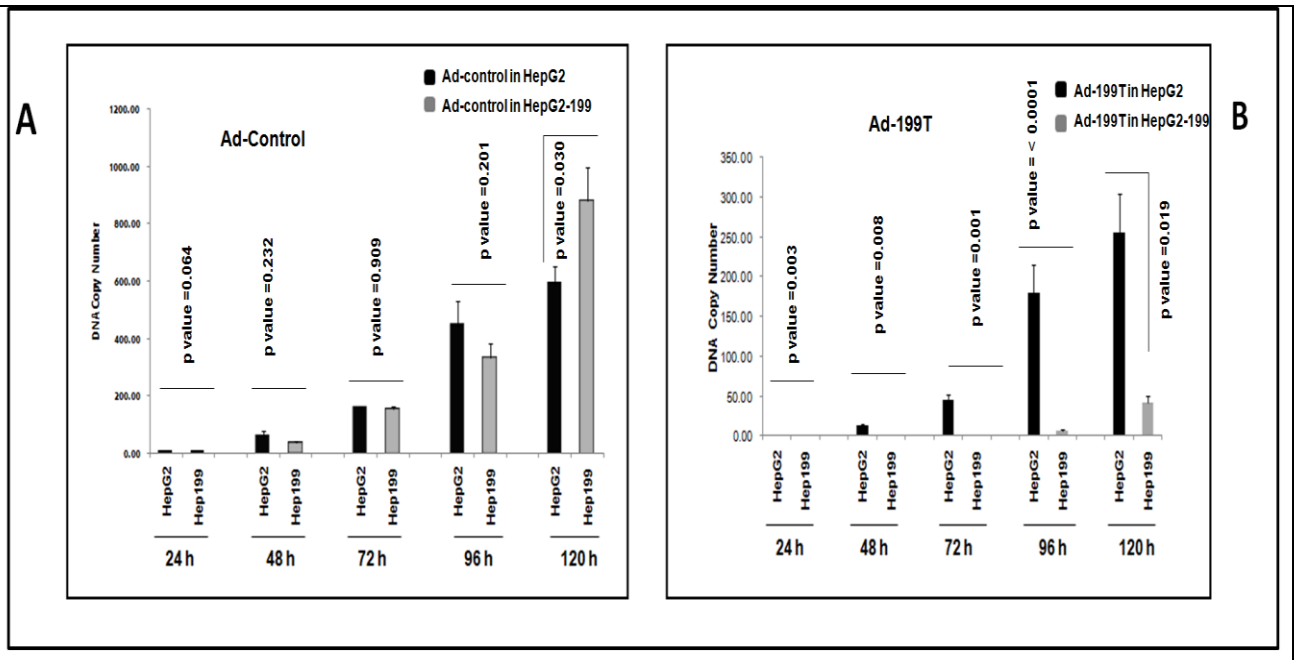


Figure 4.11 miR-199 controls Ad-199T replication *in vitro*. A Quantitative PCR results using wtAd5-11523-F/ wtAd5-11681-R primers on DNA extracted from HepG2 and HepG2-199 cells, infected with Ad-Control: active viral replication is confirmed in both cell lines by progressive accumulation of viral DNA (bar graphs) at 24, 48, 72, 96 and 120 h post infection. Each time point was analyzed in triplicate, standard deviation is indicated. **B** Quantitative PCR results using the same primers on DNA extracted from HepG2 and HepG2-199 cells, infected with Ad-199T: active viral replication is confirmed only in HepG2 cell lines by progressive accumulation of viral DNA (bar graphs) at 24, 48, 72, 96 and 120 h post infection. In HepG2-199 cells infected with Ad-199T, the viral DNA did not increase over time, confirming that the virus replication was inhibited by the presence of miR-199. Each time point was analyzed in triplicate, standard deviation is indicated.

To verify that miR-199 regulates Ad-199T replication through E1A modulation, HepG2-199 cells were infected with Ad-Control and with Ad-199T and analyzed for E1A expression 24 h post infection. For this purpose RNAs from infected cells were extracted, analyzed by electrophoresis on agarose gel (**Figure 4.12 B**), treated with DNase and reverse transcribed to cDNAs (**Figure 4.12 C**). cDNAs were then analyzed by quantitative PCR using wtAd5-11523-F/ wtAd5-11681-R primers, which allows E1A amplification. The results show that E1A expression was significantly reduced in Ad-199T infected cells, compared to control (p-value 0,011). This evidence indicates that miR-199, constitutively expressed in this cell line, controls E1A expression of Ad-199T. Conversely, E1A expression of Ad-Control virus, lacking the miR target sequence, is miR-199 independent. (**Figure 4.12 A**),

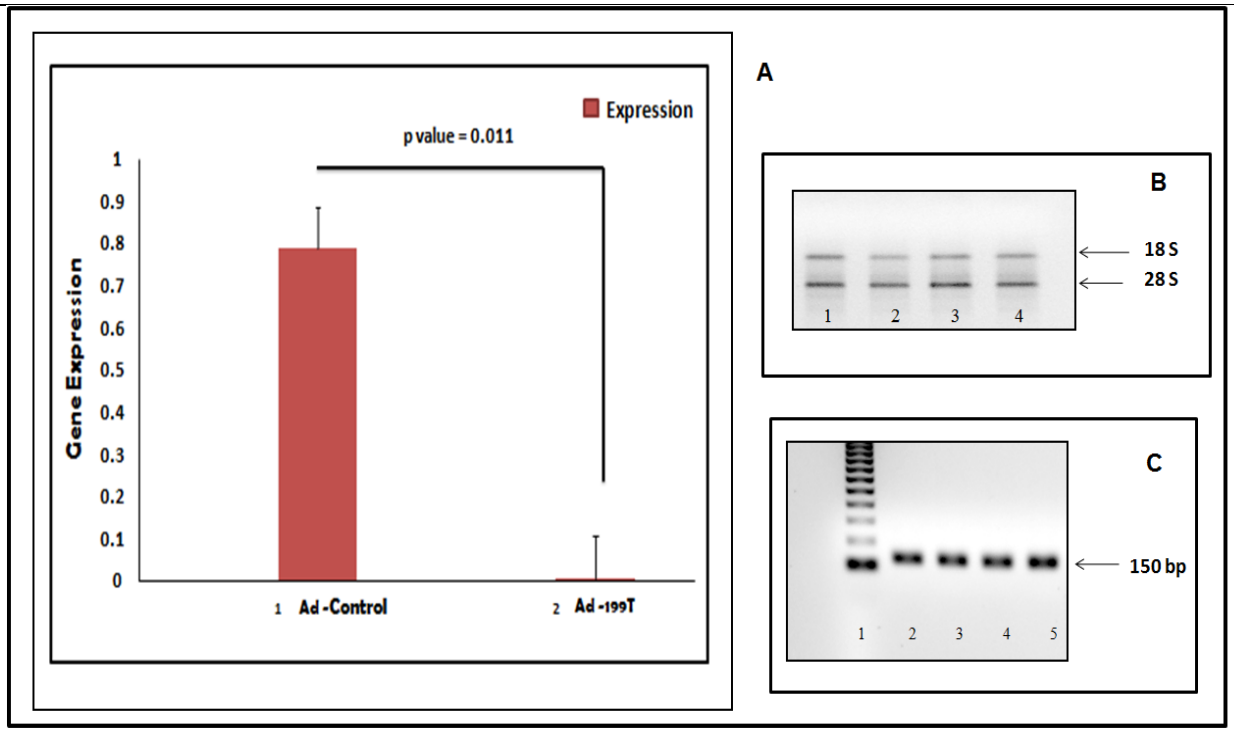


Figure 4.12 miR-199 regulates Ad-199T replication through E1A modulation. A Quantitative PCR on cDNAs from HepG2-199 cells, infected with Ad-199T and Ad-Control, using wtAd5-11523-F/ wtAd5-11681-R primers: E1A expression was significantly reduced in Ad-199T infected cells, compared to Ad-Control infected cells. Each time point was analyzed in triplicate, standard deviation is indicated. beta actin was used to normalize data. **B** Electrophoresis of RNAs extracted from: (1) non infected HepG2-199 cells, (2, 3) HepG2-199 infected with Ad-199T, and (4) HepG2-199 infected with Ad-Control. Bands corresponding to 28S and 18S rRNA are shown. **C** Electrophoresis of PCR products using B actinRNA-1249-F/ B actinRNA-1399-R primers on cDNAs derived from HepG2-199 cells: (2) non infected, (3, 4) infected with Ad-199T, and (5) infected with Ad-Control, (1) 123 bp molecular weight marker. The size of the amplification product is 150 bp.

To assess the different expression of E1A protein from Ad-199T in absence or in presence of miR-199, HepG2 and HepG2-199 cells were infected with Ad-199T and with Ad-Control, and analyzed for the presence of E1A protein. For this purpose, cell crude extract from infected cells were analyzed by western blot using a polyclonal anti-E1A antibody The results show that, in HepG2 cells, E1A protein was expressed and detectable following infection with both viruses, confirming that, in absence of miR-199, both viruses were competent for replication. Conversely, in HepG2-199, the E1A protein was detectable only in Ad-Control infected cells, while in Ad-199T infected cells the protein was absent. This evidence confirms that miR-199, constitutively expressed in this cell line, controls E1A expression of Ad-199T, while E1A expression of Ad-Control virus, lacking the miR target sequence, is miR-199 independent. (**Figure 4.13**), Based on the results, Ad-199T replication was thus significantly suppressed in miR-199 expressing cells, while Ad-Control virus was still able to replicate in these cells.

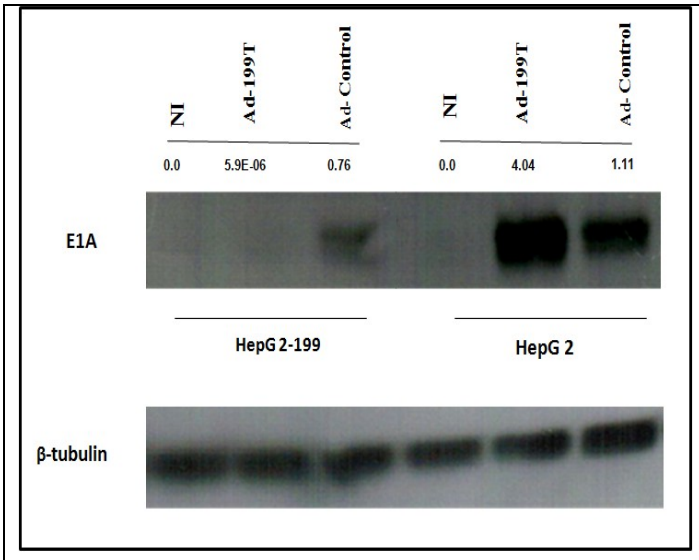


Figure 4.13 miR-199 controls E1A expression of Ad-199T. Western blot analysis of E1A protein from HepG2-199 and HepG2 cells: cells were either non infected (NI); infected with Ad-199T (Ad-199T); or infected with Ad-Control (Ad-Control). β-tubulin protein was used to normalize data with Image J software analysis. The result indicates that miR-199, constitutively expressed in HepG2-199 cells, controls E1A expression of Ad-199T.

To assess replication properties of recombinant adenoviruses *in vivo,* we assessed the ability of Ad-199T virus to replicate in the liver of B6D2 wild type mice. Ten millions v.p. of the Ad-199T virus or $1x10^7$ v.p. of the Ad-Control virus were intra-hepatically injected into 4 days old mice. At 72 hours after infection, livers were collected and viral DNA quantified using semi-quantitative and quantitative PCR (**Figure 4.14**). The results demonstrated that the viral DNA was strongly reduced in livers of mice infected with Ad-199T virus in comparison with livers of mice treated with Ad-Control (**Figure 4.14 A**). These results were confirmed by quantitative Real Time PCR (p-value =

0.054) (**Figure 4.14 B**). These findings demonstrated that miR-199 could control Ad-199T replication in normal liver cells.

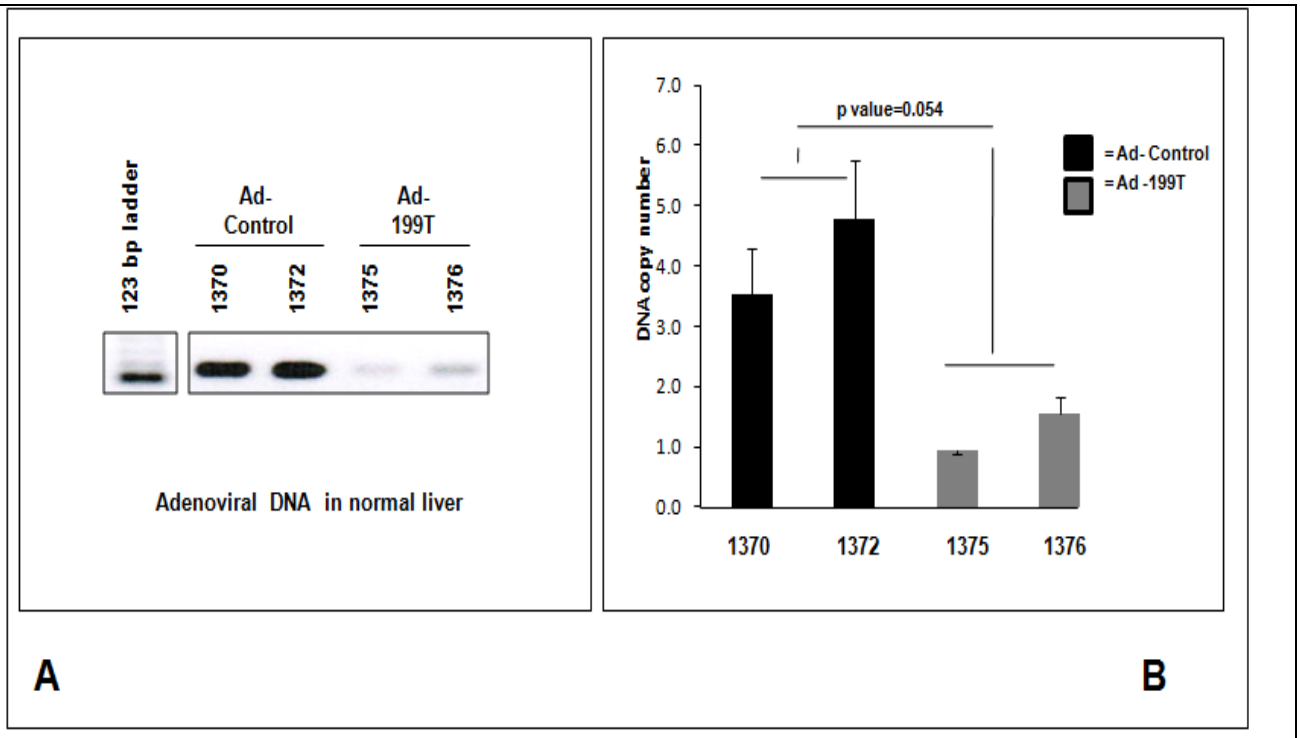


Figure 4.14 miR-199 controls Ad-199T replication in normal liver cells. **A**. Electrophoresis of PCR products using wtAd5-11523-F/ wtAd5-11681-R primers on genomic DNAs extracted from livers of mice injected with Ad-Control (1370, 1372) and with Ad-199T(1375, 1376). Quantitative Real Time PCR using the same primers on the same samples. The result showed that the replication of Ad-199T was significantly suppressed in normal liver comparing with Ad-Control. Each sample was analyzed in triplicate. standard deviation is indicated.

4.3 Ad-199T can eliminate tumor cells with the same efficiency of Ad-Control virus

After proving that Ad-199T virus cannot or poorly replicate in normal liver cells, we aimed at verifying whether this same virus could still replicate in tumor cells *in vivo*. Ad-199T or Ad-Control were used to infect HepG2 derived cells (HepLuc) implanted into the liver of B6D2 wild type mice.

Two millions HepLuc cells, derived from HepG2 and over-expressing the Luciferase reporter gene, were intra-hepatically implanted in each of eighteen B6D2 wild type mice at 3 days after birth. To

verify the presence of the HepLuc cells into the target liver tissue, the mice were examined at the *In Vivo* Imaging System (IVIS) Spectrum and light emission measured two hours after cell implantation. The detection of a very strong light emission established the presence of implanted cells in the liver of all animals (**Figure 4.15 A**).

After 24 hours, three experimental groups, consisting of six mice each, were defined: one infected intra-hepatically with $1x10^7$ v.p. of the Ad-199T virus, the second with $1x10^7$ v.p. Ad-Control virus and the third group was not infected, to monitor HepLuc cells over time.

Mice were monitored at the IVIS at 24, 48 and 72 hours after virus infection. Non virally infected animals exhibited a strong signal at 24 hours, which slightly decreased at 48 and 72 hours, indicating the presence of the cells during all the observation time points. Conversely, in the virally infected animals the signal decreased more rapidly, to almost completely disappear at 72 hours. These results suggest that the implanted tumor cells were likely eliminated due to active viral replication by either Ad-Control or Ad-199T (**Figure 4.15 B, C, D**). A quantitative photon counting analysis of the region-of-interest showed a highly significant decrease (P Value <0.05) of luminescence in mice infected with the Ad-Control or the Ad-199T viruses vs. control uninfected animals (**Figure 4.15 E**).

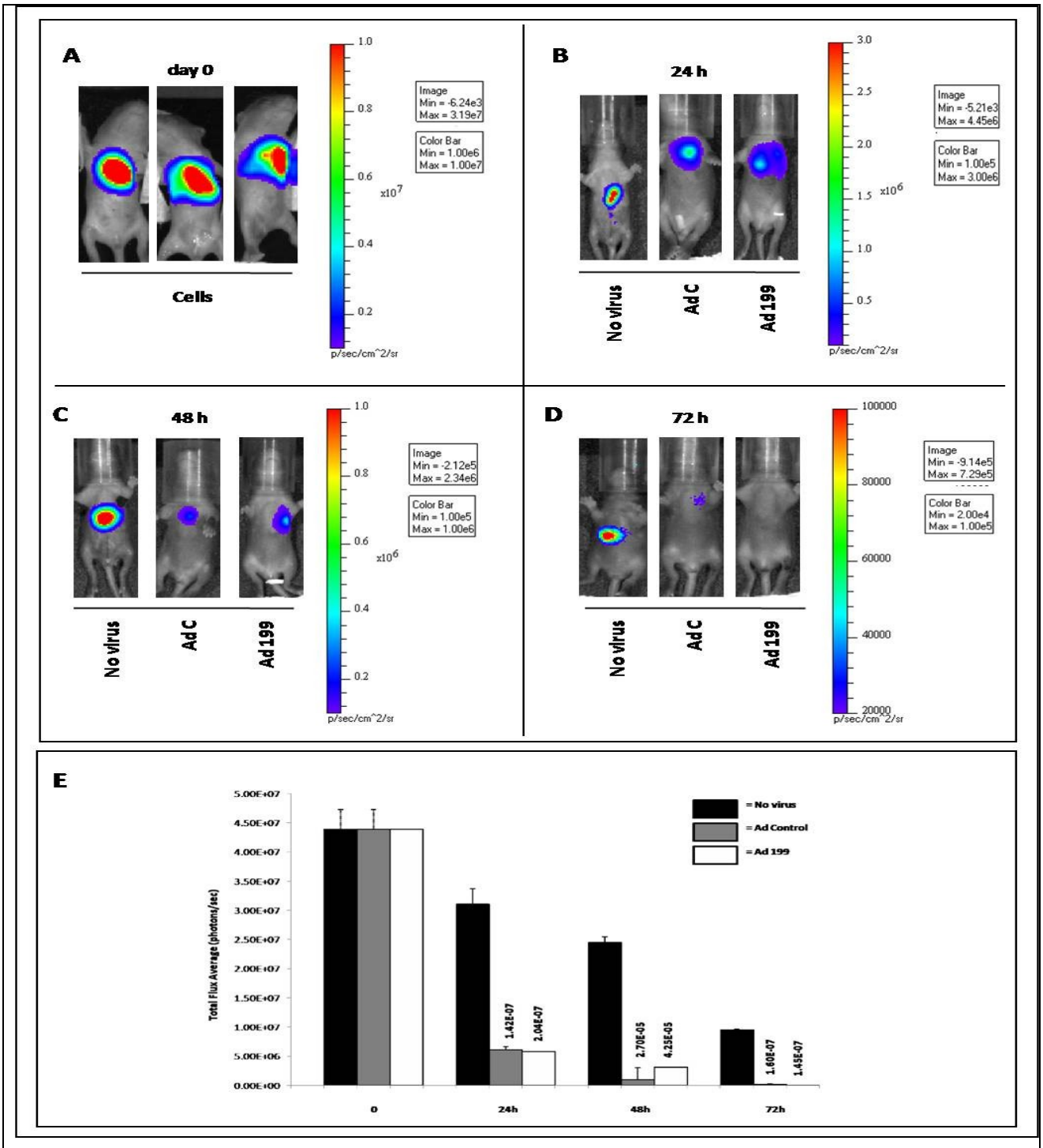


Figure 4.15 Ad-199T replicate in tumor cells *in vivo.* **A** Two millions HepLuc cells were intra-hepatically implanted in eighteen B6D2 wild type mice at 3 days after birth and examined at the *In Vivo* Imaging System (IVIS) Spectrum two hours after cell implantation. After 24 hours, the mice were infected intra-hepatically with $1x10^7$ v.p. of the Ad-199T virus (Ad-199T), or with $1x10^7$ v.p. Ad-Control virus (Ad- Control) or not infected (no virus). Mice were monitored at the IVIS at 24 hours **(B)**, 48 hours **(C)** and 72 hours **(D)** after virus infection. Reduction in pseudocolor images, representing bioluminescence intensity, indicates that the implanted tumor cells were likely eliminated due to active viral replication by either Ad-Control or Ad-199T. **E** A quantitative photon counting analysis of the region-of-interest showed a highly significant decrease (P Value <0.05) of luminescence in mice infected with the Ad-Control or the Ad-199T viruses vs. uninfected animals.

The livers of mice sacrificed at 72 hours were macroscopically analyzed. A tumor mass was evident in correspondence of the signal detected at the IVIS luminometer in non-infected animals. Conversely, in mice injected with both viruses, there was evidence of significant reduction or loss of liver tumor masses, consistent with the lack of luminous signal (**Figure 4 .16**). These data strongly suggested that both Ad-199T and Ad-Control viruses could replicate *in vivo* and eliminate the implanted tumor cells.

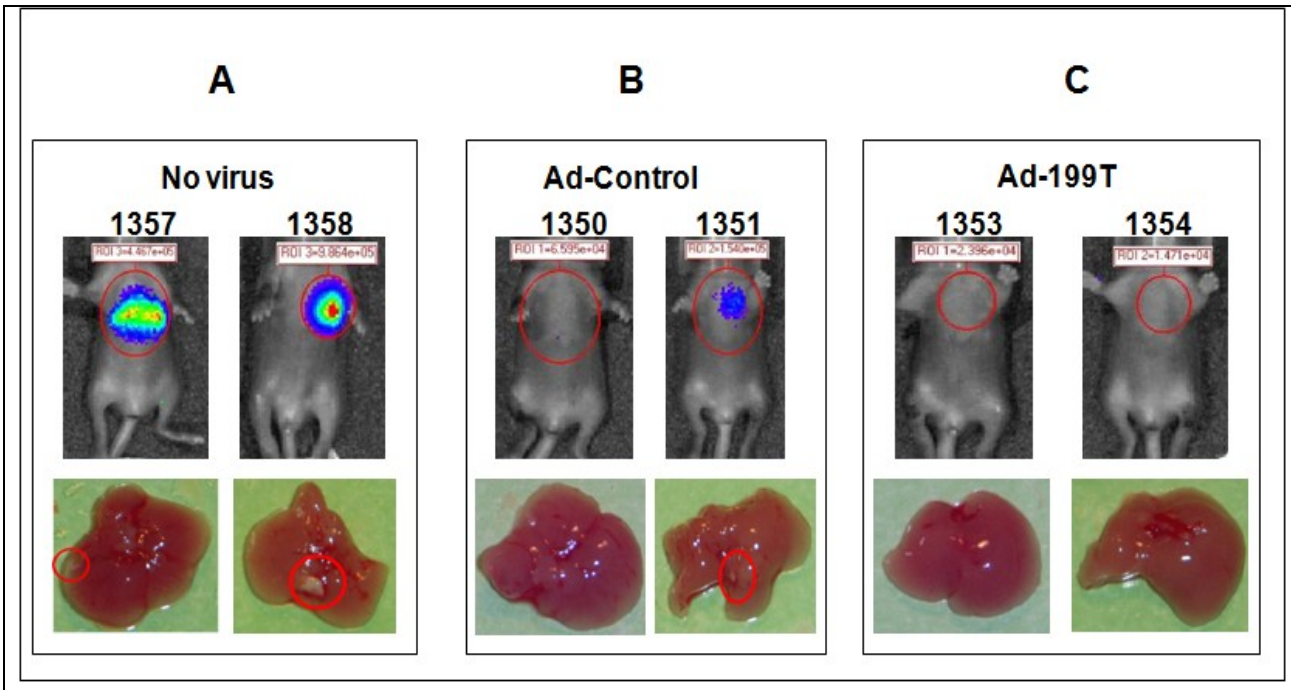


Figure 4.16 Reduction in bioluminescence intensity indicates reduction of tumor masses. At 72 hours of experiment shown in **Figure 4.15**, mice were sacrificed and livers removed. Images of livers show the presence of tumor masses whose size is approximately proportional to luminescent signals. Tumor masses are large in controls not treated with viruses and significantly reduced in mice treated with viruses.

To confirm that the Ad-199T replication was restricted to cancer cells, two millions HepLuc cells were intra-hepatically implanted in each of six B6D2 wild type mice at 3 days after birth. After 24 hours, mice were infected intra-hepatically with $1x10^7$ v.p. of the Ad-199T virus.

Mice were sacrificed at 24, 48 and 72 hours after virus injection. Livers were collected and the presence of viral DNA was assessed both in the tumors and in the surrounding normal tissues (**Figure 4.17 A**) The results show the presence of viral DNA only in the tumor tissues, indicating that active viral replication has occurred in the tumor, but not in the normal liver. The result confirms

that the replication of Ad-199T virus is significantly suppressed in normal liver but it is still effective in tumor cells (**Figure 4.17 B**).

Because normal liver cells contain significant amount of microRNA-199, these data establish that the introduction of miR-199 target sites at the E1A gene could represent a strategy for reducing hepatoxicity, but still retaining oncolytic activity of recombinant adenoviruses.

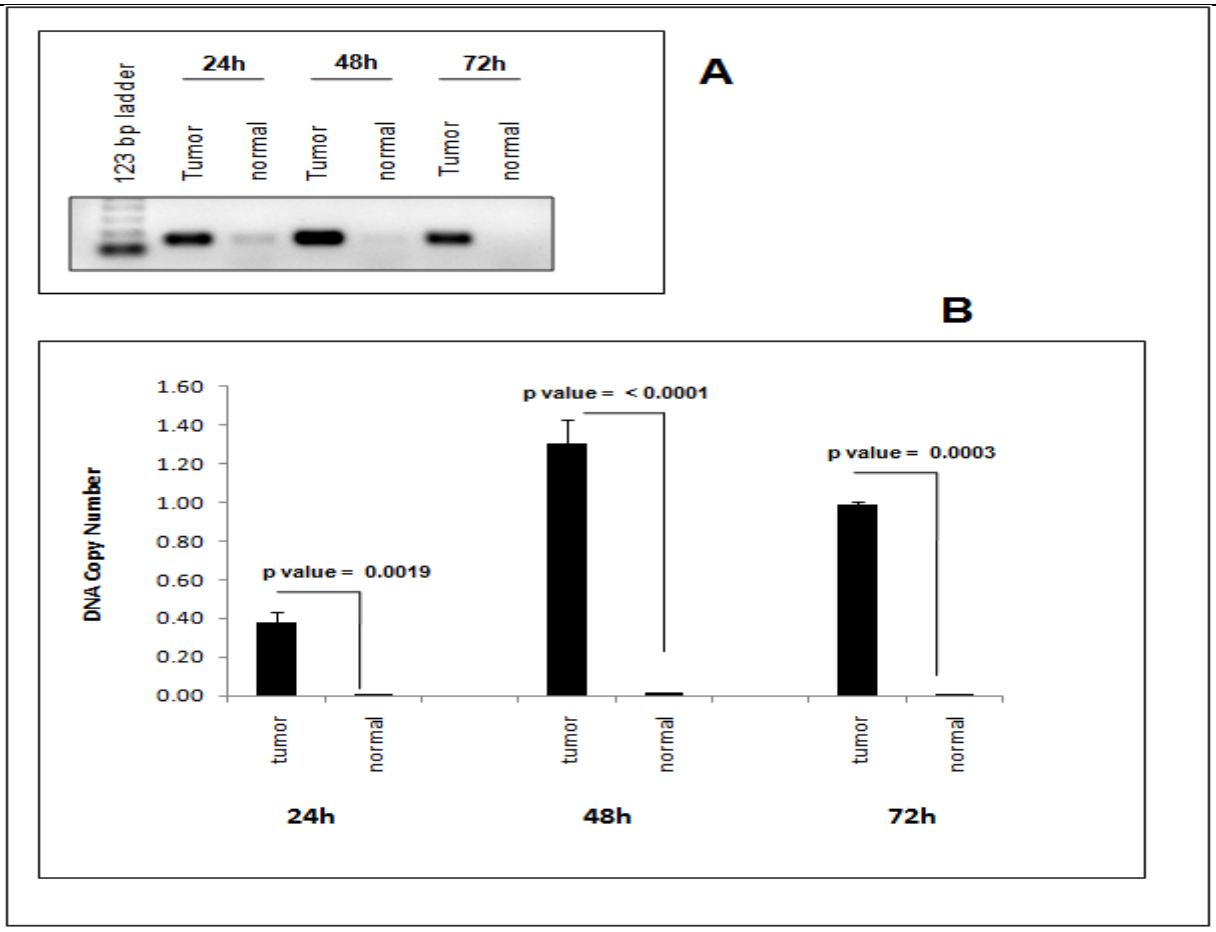


Figure 4.17 Differential replication of Ad-199T in normal liver versus tumor cells. Following liver implantation of HepLuc cells, Ad-199T virus was directly injected into the liver. Mice were sacrificed at various time-points (24h, 48h and 72 h p.i.). Adenoviral DNA was amplified from DNAs isolated from tumor masses and from surrounding normal liver. Semi-quantitative (**A**) and quantitative (**B**) PCR reactions were performed to assess viral replication. **A**. Electrophoresis of PCR products amplified using wtAd5-11523-F/ wtAd5-11681-R primers. **B**. quantitative Real Time PCR using the same primers on the same samples. The result shows a significant suppression of Ad-199T replication in normal liver compared to tumors.

5. DISCUSSION

HCC is the fifth most frequent neoplasm and the third leading cause of cancer-related death worldwide (156). In spite of general improvements in cancer treatments, survival has not significantly improved for HCC patients because of the advanced stage of the disease at diagnosis and the still limited therapeutic options. For HCC patients, complete tumor removal represents the only long-term cure. Liver resection is the choice for patients with tumors less than 5 cm in the absence of cirrhosis. In these patients, mortality rate of less than 2% can be expected (157, 158) . However, resection of the tumor by partial hepatectomy can be accomplished in a limited number of patients (generally < 15-30%) due to the extent of underlying cirrhosis. In patients with cirrhosis, the extent of liver resection that can be tolerated is significantly more limited. Among patients who undergo successful resection, 5-year survival rates is up to 74%. Following resection, however, up to 75% of patients will develop intrahepatic recurrence within 5 years (159) . Compared with resection, orthotopic liver transplantation (OLT) offers several potential advantages. Complete hepatectomy eliminates the possibility of local recurrence at the resection margin and removes the cirrhotic liver, which is clearly predisposed to tumor formation. OLT was established as the therapy of choice for patients with significant cirrhosis and limited tumor burden (160-163).

In patients who are not candidates for liver transplantation or resection, tumor ablation can be offered to extend life and to potentially downstage the tumor and permit transplantation or resection. The most commonly offered therapy is transcatheter arterial chemoembolization (TACE) (164). TACE delivers high local doses of chemotherapy, including doxorubicin, cisplatin, or mitomycin C to the tumor through the feeding artery. The impact of TACE on the clinical outcome remains unclear and is contraindicated in patients with advanced cirrhosis and hepatic decompensation.

The use of systemic chemotherapy has been attempted. Unfortunately, HCC is minimally responsive to systemic chemotherapy. Among the agents tried, doxorubicin-based regimens appear to have the greatest efficacy with response rates of 20-30% but a minimal impact on survival. More recently, the multikinase inhibitor sorafenib, able to target multiple pathways and blocking RAF/MEK/ERK signaling at the level of raf-kinase as well as by inhibiting vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor beta (PDGFR-beta), was shown to improve survival and increase time to disease progression (165, 166) . Sorafenib has become the most promising chemotherapeutic agent in the treatment of advanced HCC in patients with preserved liver function. In addition to sorafenib, sunitinib, bevacizumab, epidermal growth

factor receptor inhibitors, and mammalian target of rapamycin (mTOR) inhibitors have shown activity in small patient cohorts. In spite of all these efforts, with the exception of early and very early tumor stages, HCC remains an uncurable disease and new more effective and less toxic therapeutic agents are needed.

Oncolytic virotheraphy emerged as a promising experimental approach to fight cancer. Oncolytic Ad5-based viruses have shown efficacy and safety in preclinical (167-170) and clinical trials (102, 171) including the treatment of prostate cancer (172-174). However, variable expression of the primary Ad5 receptor, the coxsackie-adenovirus receptor, may limit the efficacy of Ad5-based constructs (175). Various approaches can be used to improve adenoviral transduction of cancer cells. For example, switching the Ad5 fiber knob to serotype 3 knob improves the transduction and enhances the cell-killing capacity of the virus in the context of many cancer types including prostate cancer (167, 170) or and colon cancer (176). Several clinical reports have indicated promising antitumor capabilities with oncolytic viruses, although there are only a few reports of complete tumor eradication. One possibility to improve therapeutic efficacy is to use the synergy of radiation and oncolytic adenoviruses (177, 178). Another possibility recently develop by combination adenoviral virotherapy and chemotherapy to eradicates malignant glioma (179). Additional clinical trials using oncolytic Ads have been carried out, and promising results have been reported (180-182) .

For safe and effective gene therapy, target tissue-restricted virus expression is desirable. Conditionally replicative Adenoviruses (CRAds) specifically aim at killing tumor cells while sparing normal cells have been introduced as new agents for cancer therapy (99, 183, 184). Various methods have been developed to achieve such selective expression.

Different types of oncolytic Adenoviruses (Ads) have been developed. The most common type of oncolytic Ads were designed to drive E1A gene expression cassette by a tumor-specific promoter. Employed tumor-specific promoters included the alpha-fetoprotein promoter (45), prostate-specific antigen promoter (46), osteocalcin promoter (47), and cyclooxygenase-2 promoter (48). The tumor-specific E1A expression renders the oncolytic Ads tumor specifically replicative; however, in any case, oncolytic viruses could also replicate in normal cells, leading to undesirable toxicity.

Another approach was based on the introduction of E1B deletions to make viral replication possible in p53-defective cells. The best known of these viruses is ONYX-015. The oncolytic adenovirus ONYX-015 was the first to be tested in clinical trials, revealing itself as a well tolerated and safe tool and so to be a promising therapeutic agent in cancer (185). Even a combination between this

virus and standard chemotherapy was demonstrated to be a strong trial to increase the antitumor activity of virotherapy (186).

Here, we developed a novel CRAd whose replication is controlled by miR-199. The virus can replicate in miR-199-negative cells. Since HCC cells typically exhibit a strong down-regulation of miR-199, which is instead expressed at substantial level in normal hepatocytes as well as in other normal tissues. Thus, this virus should be able to deliver its cytocidal effect to cancer but not to normal cells, thereby addressing the important issue of a novel therapeutic approach with improved efficacy and safety in HCC.

The methods of using miRNA target (miRT) sequences was first described by Naldini and colleagues to specifically modulate transgene expression in hematopoietic cells (146) or hepatocytes (187) . Kelly *et al* employed a similar approach to restrict replication of a coxsackievirus to inhibit its replication in normal muscle tissue and reduce muscle inflammation without compromising tumor cell-killing ability (21). More recently, Fu and colleagues constructed a LCSOV (liver-cancer-specific oncolytic virus) in which the essential viral glycoprotein H gene (gH gene) was controlled by the apoE-AAT liver-specific promoter and by the presence of complementary sequences to miR-122, miR-124a and let-7a in its 3'UTR (188). Using that strategy the authors were able to create an oncolytic virus able to kill HCC cells.

It has also been described a miR-122-detargeting approach to reduce adenovirus-induced liver toxicity (96, 97). These studies have used the wild type E1A promoter to control E1A and demonstrated that the presence of miR-122 target sequences within the 3′UTR of E1A gene could reduce E1A expression in hepatic cells and reduce hepatotoxicity.

In this work, we exploited the differential expression of miR-199 between tumor versus non-neoplastic liver tissues to modulate the expression of E1A gene and selectively permit the replication of the oncolytic virus only in HCC cells. We demonstrated this property of AD-199T both *in vitro* and *in vivo*. We demonstrated that AD-199T virus was not able to replicate in a cell line stably expressing the miR-199, while the adenoviral replication proceeded exponentially in HCC cells lacking the microRNA. To ensure that this property of AD-199T was functional *in vivo*, we administrated it intra-hepatically in 3 days old mice, thus avoiding the confounding effect that would be produced by immune system reaction (189) . Also in this *in vivo* model, the AD-199T was not able to replicate, in spite of the fact that an identical control virus, lacking the miR-199 target sites, could efficiently undergo several rounds of replication.

To give support to the oncolytic activity of AD-199T, we proved that the virus, was able to inhibit the growth of tumor cells previously implanted in the liver with the same efficiency of Adeno control virus. The presence of AD-199T viral DNA only in neoplastic tissues and the absence of detectable amount of viral DNA in normal liver indicated the ability of the miR-199 target sequences to inhibit adenoviral replication and so precisely direct the oncolytic activity against tumor cells. This work is the first that make use of miR-199 to target an oncolytic virus to cancer cells. Other works have utilized microRNAs to reduce viral pathogenicity in normal tissues (190-192).

These results suggest the knowledge of miRNA expression in normal and cancer cells may be used to design viruses with two properties: efficacy against cancer cells combined with minimal adverse side effects. The demonstration that HCC can be selectively targeted with little or no toxicity to normal hepatocytes suggest that this is a feasible task and other therapeutic options may potentially exist.

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Tutore: Prof. (Cognome e Nome)

Sabbioni Silvia

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